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(54) Title: BRAIN SPECIFIC BINDING MEMBERS

(57) Abstract: The present invention relates to specific binding members directed to the brain, including specific binding members able to pass through the blood brain barrier or which are directed to areas of brain inflammation or blood brain barrier breakdown. Panels and mixtures of antibodies are provided, also individual antibody molecules and VH and VL domains, along with methods of obtaining specific binding members by selection, e.g. using libraries displayed on the surface of filamentous bacteriophage.

BRAIN SPECIFIC BINDING MEMBERS

The present invention relates to specific binding members directed to the brain, including specific binding members able to pass through the blood brain barrier or which are directed to areas of brain inflammation or blood brain barrier breakdown. Panels and mixtures of antibody are provided, also individual antibody molecules and VH and VL domains, along with methods of obtaining specific binding members by selection using libraries displayed on the surface of filamentous bacteriophage.

Transport of many blood-borne molecules into the brain is restricted when compared to other tissues in the body. This has led to the concept of a blood brain barrier (BBB) which isolates the brain from peripheral changes in physiology (Bradbury MWB (1993) Experimental Physiology, 78: 453-472). It has been shown that the endothelial cells which line the capillaries of the brain are joined by extensive tight junctions which restrict pericellular movement of water-soluble molecules. These endothelial cells also express specific transporter molecules on their surfaces which selectively allow the passage of molecules which are essential for brain function e.g. glucose, amino acids, transferrin.

The BBB presents a substantial hurdle for the delivery of many

drugs, particularly proteins such as antibodies and cytokines or growth factors into the central nervous system. With the exception of lipid-soluble molecules, which have a molecule weight from 400-600 Da, virtually all drugs that originate from either biotechnology or classical small molecule pharmacology undergo negligible transport through the intact BBB. The identification of novel transporter molecules or receptors associated with the BBB represents a potential target for enhancing the uptake of drugs into the CNS, by for example conjugation to the natural ligands of those receptors or by selection of antibodies or drugs which bind directly to the receptors.

Central Nervous System (CNS) diseases that may be treated in this manner include sleep disorders (e.g. narcolepsy), affective disorders, schizophrenia, regenerative disorders such as Alzheimer's disease, Parkinson's disease, fronto-temporal dementia, Huntington's chorea, demyelination diseases (e.g. multiple sclerosis), spinal cord injury, brain tumours (primary and secondary), viral encephalopathies (e.g. rabies and cerebral HIV), motor neurone diseases, prion diseases, hydrocephalus, stroke (primary haemorrhagic and ischaemic), epilepsies, obsessive-compulsive disorders, anxieties and phobias, drug abuse, alcohol and other substance abuses, symptoms such as pain. In addition, consciousness may be modulated, such as in general anaesthesia.

The present invention relates to the selection and characterisation of human antibodies from a phage display antibody library which have been demonstrated to cross the BBB or which localise to areas of inflammation in brain tissue. Such antibodies have value and therapeutic potential as agents to enhance drug delivery across the blood brain barrier, and as tools to identify novel transporter or other blood brain barrier or inflamed tissues marker molecules which may themselves be targets for therapeutic intervention.

Some molecules on brain capillary endothelium are already known to be specific receptors for circulating proteins or plasma proteins. Human microvessels have been used to characterise the human BBB receptors for insulin, insulin-like growth factor I, insulin-like growth factor-II, transferrin and leptin. (Reviewed by W Pardridge (1997) Journal of Cerebral Blood Flow and Metabolism, 17: 713-731). The BBB insulin, insulin-like growth factor, and transferrin receptor systems have been shown to mediate the transcytosis of peptides through the BBB *in vivo* (e.g. Duffy and Pardridge, 1987, Brain Res. 420: 32-38). The observation that peptide receptors are present on brain capillaries endothelium, and that some of these mediate peptide transcytosis through the BBB led to the idea of covalently coupling nontransportable drugs to a protein or peptide that normally undergoes

receptor-mediated transcytosis through the BBB. Initial experiments were carried out using cationized albumin which undergoes absorptive-mediated transcytosis through the BBB (Kumagai et al., 1987, J Biol. Chem 262: 15214-15219). Subsequently murine mAbs to the rat transferrin receptor (Friden et al., 1993, Science 259: 373-377) and the human insulin receptor were developed (Pardrige et al., 1995, Pharm Res 12: 807-816) which have BBB permeability.

The anti-human insulin receptor mAb 83-14 has been conjugated to streptavidin and shown to retain its ability to be transported (Wu et al, 1997, J Clin Invest, 100: 1804-12) allowing biotinylated compounds to be transported along with it.

Little is known about the up or down-regulation of transport systems at the BBB in disease states, but there are indications that change at the level of expression occurs e.g. the expression of ICAM-1 and ICAM-2 is upregulated in areas of inflammation (Osborn, L. Cell 1990, 623-6). Following stimulation with lipopolysaccharide and interleukin -1 and -6 lymphocyte binding to the BBB was increased 3-fold and could be blocked by mAbs specific for VLA4, CD18 and CD11a (de Vries et al., J. Neuroimmunol 1994, 52; 1-8). Thus disease states can yield important pointers towards achieving selectivity in BBB transport by identifying up-regulated transport systems at

the BBB. The picture that is emerging from *in vitro* and *in vivo* studies in animal modes is that the cross-linking of cell-surface adhesion molecules initiates an intracellular signalling cascade that leads to changes in the cytoskeleton, disassembly of the junctional complex and, ultimately, an increase in vessel permeability.

In one aspect, the present invention provides a mixture or panel of at least 5, and preferably at least 10 different specific binding members each comprising an antibody VH variable domain and/or an antibody VL variable domain, wherein an antibody VH variable domain has an amino acid sequence selected from the group consisting of the G65 (SEQ ID NO. 2), G67 (SEQ ID NO. 6), G73 (SEQ ID NO. 10), G76 (SEQ ID NO. 14), G77 (SEQ ID NO. 18), G78 (SEQ ID NO. 22), G79 (SEQ ID NO. 24), G81 (SEQ ID NO. 30), G83 (SEQ ID NO. 34), G85 (SEQ ID NO. 38), G88 (SEQ ID NO. 42), G92 (SEQ ID NO. 46), G93 (SEQ ID NO. 50), G95 (SEQ ID NO. 54), G101 (SEQ ID NO. 58), G102 (SEQ ID NO. 62), G110 (SEQ ID NO. 66) and G112 (SEQ ID NO. 70) VH domain sequences disclosed herein, and/or an antibody VL variable domain has an amino acid sequence selected from the group consisting of the G65 (SEQ ID NO. 4), G67 (SEQ ID NO. 8), G73 (SEQ ID NO. 12), G76 (SEQ ID NO. 16), G77 (SEQ ID NO.20), G78 (SEQ ID NO. 24), G79 (SEQ ID NO. 28), G81 (SEQ ID NO. 32), G83 (SEQ ID NO. 36), G85 (SEQ ID NO. 40), G88 (SEQ ID NO. 44), G92 (SEQ ID NO. 48), G93 (SEQ ID NO. 52), G95 (SEQ ID NO. 56),

G101 (SEQ ID NO. 60), G102 (SEQ ID NO. 64), G110 (SEQ ID NO. 68) and G112 (SEQ ID NO. 72) VL domain sequences disclosed herein, each specific binding member being able, when displayed on the surface of filamentous bacteriophage particles, to pass through a mammalian blood brain barrier, preferably a human or rodent (e.g. rat) blood brain barrier. The blood brain barrier may be an *in vitro* barrier such as generated in accordance with Example 1 herein.

A mixture or panel of specific binding members may include at least 11, 12, 13, 14, 15, 16, 17 or 18 of the VH or VL domains. In such a mixture, preferably VH and VL domains are paired according to corresponding nomenclature as used herein (e.g. G65 VH (SEQ ID NO. 2) with G65 VL (SEQ ID NO. 4), G81 VH (SEQ ID NO. 30) with G81 VL (SEQ ID NO. 32), and so on).

A panel or mixture of specific binding members provided by the present invention is useful for selection of specific binding members that cross the blood brain barrier. Additionally, a panel or mixture may be employed together to deliver different molecules or effector functions.

One or more specific binding members may be selected from a panel or mixture e.g. by binding with antigen, which may be on endothelial cells. Where the specific binding members are displayed on filamentous bacteriophage, this allows nucleic

acid encoding a selected specific binding member, or at least a component of the member, to be readily isolated. Encoding nucleic acid may then be manipulated and may be used to produce more of the specific binding member or component thereof by means of recombinant expression.

In a further, general aspect, the present invention provides a method of obtaining one or more specific binding members, the method including bringing into contact a library of specific binding members displayed on the surface of filamentous bacteriophage particles, and selecting one or more specific binding members of the library able when displayed on filamentous bacteriophage particles to pass through a mammalian blood brain barrier. Selection on ability to pass through a blood brain barrier may be followed by one or more further rounds of selection for ability to bind a type of cell of the brain or CNS, for instance endothelial cells, or cells of the meninges, parenchyma, choroid plexus, cerebrum, cerebellum, spinal cord or microglia.

A population or mixture of specific binding members which when displayed on filamentous bacteriophage are able to pass through a mammalian blood brain barrier is obtainable on selection from a phage display library as disclosed. Such a population or mixture may comprise at least 10^6 different specific binding members. One or more further rounds of

selection may be used to provide a sub-population of about 10,000 different specific binding members, 1,000, 100, a mixture of 10-20 different specific binding members or fewer, and individual specific binding members.

The invention further provides a plurality of different antibody VH variable domains obtainable from a mixture, panel, population or library as disclosed, and further provides a plurality of different antibody VL variable domains obtainable from such a mixture, panel, population or library.

Individual VH and VL domains of which sequences are shown herein each represent individual aspects of the present invention. A VH domain of which the sequence is disclosed herein may be combined with a VL domain of which the sequence is disclosed herein, or other VL domain, to provide a VH/VL pairing representing an antigen-binding site of an antibody. Similarly, a VL domain of which the sequence is disclosed herein may be combined with a VH domain of which the sequence is disclosed herein, or other VH domain. Pairings of VH and VL domains represent further aspects of the present invention. Preferred pairings are as identified by corresponding nomenclature herein, e.g. G65 VH (SEQ ID NO. 2) with G65 VL (SEQ ID NO. 4), G81 VH (SEQ ID NO. 30) with G81 VL (SEQ ID NO. 32), and so on.

Thus, individual aspects of the present invention provide a

specific binding member comprising a VH/VL pairing selected from the group consisting of G65, G67, G73, G76, G77, G78, G79, G81, G83, G85, G88, G92, G93, G95, G101, G102, G110 and G112 (SEQ ID NO's are given above). Each VH domain and each VL domain of each of these pairings represents a further aspect of the present invention. Of the VH/VL pairings disclosed herein, G67, G73, G76, G78, G79, G83, G85, G88, G92, G93, G95 and G110 represent preferred embodiments, being cross-reactive with human tissue.

These as phage antibodies actively cross the blood brain barrier and specifically recognise endothelial cells of the brain, as shown by ELISA and immunocytochemistry. G93 (VH SEQ ID NO. 50; VL SEQ ID NO. 52) and G73 (VH SEQ ID NO. 10; VL SEQ ID NO. 12) are among particularly preferred embodiments of the present invention; they show high levels of transport across the blood brain barrier and cross react with antigens on human endothelial cells.

As noted, further VH and VL domains, and pairings thereof, may be obtained from a filamentous bacteriophage library, employing selection for ability to pass through a blood brain barrier.

Further aspects of the invention provide the D5 VH (SEQ ID NO. 74) and VL (SEQ ID NO. 76) domains of which the amino acid

sequences are disclosed herein. D5 VH/VL is directed against human serum amyloid protein (SAP) which passes through the blood brain barrier. Experimental evidence included below shows that D5 is actively transported across the blood brain barrier.

One or more CDRs may be taken from any VH or VL domain according to the present invention and incorporated into a suitable framework. This is discussed further below.

Variants of the VH and VL domains and CDRs of which the sequences are set out herein and which can be employed in specific binding members for brain and/or endothelial cell antigens can be obtained by means of methods of sequence alteration or mutation and screening. Such methods are also provided by the present invention.

In addition to antibody sequences, the specific binding member may comprise other amino acids, e.g. forming a peptide or polypeptide, such as a folded domain, or to impart to the molecule another functional characteristic in addition to ability to bind antigen. Specific binding members of the invention may carry a detectable label, or may be conjugated to a toxin or enzyme (e.g. via a peptidyl bond or linker). Thus a VH domain or a VL domain according to the present invention may be provided in a fusion with additional amino

acids.

A further aspect of the present invention provides a method of obtaining one or more specific binding members with a desired property, the method including bringing into contact a library or panel of specific binding members and selecting one or more with the desired property. Such a method may employ phage display technology, wherein the specific binding members in the library or panel are displayed on the surface of bacteriophage particles, each particle containing nucleic acid encoding the specific binding member or a component thereof (e.g. VH domain). Nucleic acid may be taken from a bacteriophage particle containing nucleic acid encoding a selected specific binding member or component thereof, and nucleic acid with the sequence of the nucleic acid from the particle can be used to provide (by means of recombinant technology) the encoded product, or further nucleic acid with the sequence, or a variant or derivative.

In further aspects, the invention provides an isolated nucleic acid which comprises a sequence encoding a specific binding member as defined above, and methods of preparing specific binding members of the invention which comprise expressing said nucleic acids under conditions to bring about expression of said binding member, and recovering the binding member.

Specific binding members according to the invention may be used in a method of treatment or diagnosis of the human or animal body, such as a method of treatment (which may include prophylactic treatment) of a disease or disorder in a human patient which comprises administering to said patient an effective amount of a specific binding member of the invention. The specific binding member may be unconjugated, or may be conjugated to an active agent. Conditions treatable in accordance with the present invention include neurological diseases, including Alzheimer's disease, prion diseases, AIDS-related dementia, and any disease involving inflammation occurring within the brain or CNS.

These and other aspects of the invention are described in further detail below.

TERMINOLOGY

Specific binding member

This describes a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its surface, or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member

of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate. This application is concerned with antigen-antibody type reactions.

Antibody

This describes an immunoglobulin whether natural or partly or wholly synthetically produced. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic subclasses; fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or other cell producing an antibody may be subject

to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL

domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, **242**, 423-426, 1988; Huston et al, PNAS USA, **85**, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al, Proc. Natl. Acad. Sci. USA **90** 6444-6448, 1993). Fv, scFv or diabody molecules may be stabilised by the incorporation of disulphide bridges linking the VH and VL domains (Y. Reiter et al, Nature Biotech, **14**, 1239-1245, 1996). Minibodies comprising a scFv joined to a CH3 domain may also be made (S. Hu et al, Cancer Res., **56**, 3055-3061, 1996).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (e.g. by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be

conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. **4**, 446-449 (1993)), e.g. prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in *E.coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by knobs-into-holes engineering (J. B. B. Ridgeway et al, Protein Eng., **9**, 616-621, 1996).

Antigen binding domain

This describes the part of an antibody which comprises the area which specifically binds to and is complementary to part

or all of an antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains (e.g. a so-called Fd antibody fragment consisting of a VH domain). Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

Specific

This may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner(s). The term is also applicable where e.g. an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific binding member carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

Comprise

This is generally used in the sense of include, that is to say permitting the presence of one or more additional features or components.

Isolated

This refers to the state in which specific binding members of

the invention, or nucleic acid encoding such binding members, will be in accordance with the present invention. Members and nucleic acid will be free or substantially free of material with which they are naturally associated such as other polypeptides or nucleic acids with which they are found in their natural environment, or the environment in which they are prepared (e.g. cell culture) when such preparation is by recombinant DNA technology practised *in vitro* or *in vivo*. Members and nucleic acid may be formulated with diluents or adjuvants and still for practical purposes be isolated - for example the members will normally be mixed with gelatin or other carriers if used to coat microtitre plates for use in immunoassays, or will be mixed with pharmaceutically acceptable carriers or diluents when used in diagnosis or therapy. Specific binding members may be glycosylated, either naturally or by systems of heterologous eukaryotic cells (e.g. CHO or NS0 (ECACC 85110503) cells, or they may be (for example if produced by expression in a prokaryotic cell) unglycosylated.

By "substantially as set out" it is meant that the relevant CDR or VH or VL domain of the invention will be either identical or highly similar to the specified regions of which the sequence is set out herein. By "highly similar" it is contemplated that from 1 to 5, preferably from 1 to 4 such as 1 to 3 or 1 or 2, or 3 or 4, substitutions may be made in the

CDR and/or VH or VL domain.

The structure for carrying a CDR of the invention will generally be of an antibody heavy or light chain sequence or substantial portion thereof in which the CDR is located at a location corresponding to the CDR of naturally occurring VH and VL antibody variable domains encoded by rearranged immunoglobulin genes. The structures and locations of immunoglobulin variable domains may be determined by reference to (Kabat, E.A. et al, Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (http://immuno.bme.nwu.edu)).

Preferably, a CDR amino acid sequence substantially as set out herein is carried as a CDR in a human variable domain or a substantial portion thereof. The VL CDR 3 and especially the VH CDR3 sequences substantially as set out herein (e.g. see Table 4) represent preferred embodiments of the present invention and it is preferred that each of these is carried as a respective VL CDR3 or VH CDR3 in a respective human light or heavy chain variable domain or a substantial portion thereof.

Variable domains employed in the invention may be derived from any germline or rearranged human variable domain, or may be a synthetic variable domain based on consensus sequences of

known human variable domains. A CDR-derived sequences of the invention (e.g. CDR3) may be introduced into a repertoire of variable domains lacking a CDR (e.g. CDR3), using recombinant DNA technology.

For example, Marks *et al* (*Bio/Technology*, 1992, 10:779-783) describe methods of producting repertoires of antibody variable domains in which consensus primers directed at or adjacent to the 5' end of the variable domain area are used in conjunction with consensus primers to the third framework region of human VH genes to provide a repertoire of VH variable domains lacking a CDR3. Marks *et al* further describe how this repertoire may be combined with a CDR3 of a particular antibody. Using analogous techniques, the CDR3-derived sequences of the present invention may be shuffled with repertoires of VH or VL domains lacking a CDR3, and the shuffled complete VH or VL domains combined with a cognate VL or VH domain to provide specific binding members of the invention. The repertoire may then be displayed in a suitable host system such as the phage display system of WO92/01047 so that suitable specific binding members may be selected. A repertoire may consist of from anything from 10^4 individual members upwards, for example from 10^6 to 10^8 or 10^{10} members.

Analogous shuffling or combinatorial techniques are also disclosed by Stemmer (*Nature*, 1994, 370:389-391), who

describes the technique in relation to a β-lactamase gene but observes that the approach may be used for the generation of antibodies.

A further alternative is to generate novel VH or VL regions carrying a CDR-derived sequences of the invention using random mutagenesis of one or more selected VH and/or VL genes to generate mutations within the entire variable domain. Such a technique is described by Gram *et al* (1992, *Proc. Natl. Acad. Sci., USA,* 89:3576-3580), who used error-prone PCR.

Another method which may be used is to direct mutagenesis to CDR regions of VH or VL genes. Such techniques are disclosed by Barbas *et al*, (1994, *Proc. Natl. Acad. Sci., USA,* 91:3809-3813) and Schier *et al* (1996, *J. Mol. Biol.* 263:551-567).

All the above described techniques are known as such in the art and in themselves do not form part of the present invention. The skilled person will be able to use such techniques to provide specific binding members of the invention using routine methodology in the art.

A further aspect of the invention provides a method for obtaining an antibody antigen binding domain with a desired property, e.g. ability to cross the blood brain barrier and/or specificity for a brain and/or endothelial cell antigen, the method comprising providing by way of addition, deletion,

substitution or insertion of one or more amino acids in the amino acid sequence of a VH domain set out herein a VH domain which is an amino acid sequence variant of the VH domain, combining the VH domain thus provided with one or more VL domains, and testing the VH/VL combination or combinations for to identify an antibody antigen binding domain with the desired property, e.g. ability to cross the blood brain barrier and/or specificity for the antigen, and optionally with one or more of preferred properties, e.g. ability to bind areas of inflammation in the brain, breakdown of the blood brain barrier. Said VL domain may have an amino acid sequence which is substantially as set out herein.

An analogous method may be employed in which one or more sequence variants of a VL domain disclosed herein are combined with one or more VH domains.

A further aspect of the invention provides a method of preparing a specific binding member with a desired property, which method comprises:

(a) providing a starting repertoire of nucleic acids encoding a VH domain which either include a CDR3 to be replaced or lack a CDR3 encoding region;

(b) combining said repertoire with a donor nucleic acid encoding an amino acid sequence substantially as set out herein for a VH CDR3 such that said donor nucleic acid is

inserted into the CDR3 region in the repertoire, so as to provide a product repertoire of nucleic acids encoding a VH domain;

(c) expressing the nucleic acids of said product repertoire;

(d) selecting a specific binding member with the desired property; and

(e) recovering said specific binding member or nucleic acid encoding it.

Again, an analogous method may be employed in which a VL CDR3 of the invention is combined with a repertoire of nucleic acids encoding a VL domain which either include a CDR3 to be replaced or lack a CDR3 encoding region.

Similarly, one or more, or all three CDRs may be grafted into a repertoire of VH or VL domains which are then screened for a specific binding member or specific binding members with the desired property.

As noted, the desired property may be any one or more of ability to cross the blood brain barrier, bind an endothelial cell or other brain cell antigen, bind areas of inlammation in the brain or blood brain barrier breakdown, bind ICAM or bind transferrin receptor.

A substantial portion of an immunoglobulin variable domain

will comprise at least the three CDR regions, together with their intervening framework regions. Preferably, the portion will also include at least about 50% of either or both of the first and fourth framework regions, the 50% being the C-terminal 50% of the first framework region and the N-terminal 50% of the fourth framework region. Additional residues at the N-terminal or C-terminal end of the substantial part of the variable domain may be those not normally associated with naturally occurring variable domain regions. For example, construction of specific binding members of the present invention made by recombinant DNA techniques may result in the introduction of - or C-terminal residues encoded by linkers introduced to facilitate cloning or other manipulation steps. Other manipulation steps include the introduction of linkers to join variable domains of the invention to further protein sequences including immunoglobulin heavy chains, other variable domains (for example in the production of diabodies) or protein labels as discussed in more details below.

Although in a preferred aspect of the invention specific binding members comprising a pair of VH and VL domains are preferred, single binding domains based on either VH or VL domain sequences form further aspects of the invention. It is known that single immunoglobulin domains, especially VH domains, are capable of binding target antigens in a specific manner.

In the case of either of the single chain specific binding domains, these domains may be used to screen for complementary domains capable of forming a two-domain specific binding member with the desired property.

This may be achieved by phage display screening methods using the so-called hierarchical dual combinatorial approach as disclosed in WO 92/01047 in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain specific binding member is selected in accordance with phage display techniques such as those described in that reference. This technique is also disclosed in Marks *et al, ibid.*

Specific binding members of the present invention may further comprise antibody constant regions or parts thereof. For example, a VL domain may be attached at its C-terminal end to antibody light chain constant domains including human Cκ or Cλ chains, preferably Cλ chains. Similarly, a specific binding member based on a VH domain may be attached at its C-terminal end to all or part of an immunoglobulin heavy chain derived from any antibody isotype, e.g. IgG, IgA, IgE and IgM and any of the isotype sub-classes, particularly IgG1 and IgG4. IgG4 is preferred.

Antibodies of the invention may be labelled with a detectable or functional label. Detectable labels include radiolabels such as ^{131}I or ^{99}Tc, which may be attached to antibodies of the invention using conventional chemistry known in the art of antibody imaging. Labels also include enzyme labels such as horseradish peroxidase. Labels further include chemical moieties such as biotin which may be detected via binding to a specific cognate detectable moiety, e.g. labelled avidin.

Antibodies of the present invention are designed to be used in methods of diagnosis or treatment in human or animal subjects, preferably human.

Accordingly, further aspects of the invention provide methods of treatment comprising administration of a specific binding member as provided, pharmaceutical compositions comprising such a specific binding member, and use of such a specific binding member in the manufacture of a medicament for administration, for example in a method of making a medicament or other composition comprising formulating the specific binding member with at least one additional component, such as a pharmaceutically acceptable excipient.

In accordance with the present invention, compositions provided may be administered to individuals. Administration

is preferably in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, eg decisions on dosage etc, is within the responsibility of general practioners and other medical doctors. Appropriate doses of antibody are well known in the art; see Ledermann J.A. et al. (1991) Int J. Cancer 47: 659-664; Bagshawe K.D. et al. (1991) Antibody, Immunoconjugates and Radiopharmaceuticals 4: 915-922.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially, dependent upon the condition to be treated.

Antibodies of the present invention may be administered to a patient in need of treatment via any suitable route, usually by intravenous injection into the bloodstream. The precise dose will depend upon a number of factors, including whether the antibody is for diagnosis or for treatment, the size and location of the area to be treated (e.g. wound), the precise nature of the antibody (e.g. whole antibody, fragment or diabody), and the nature of any detectable label or other molecule attached to the antibody. A typical antibody dose

will be in the range 0.5mg to 100g for systemic applications, and 10μg to 1mg for local applications. Typically, the antibody will be a whole antibody, preferably the IgG4 subclass. This is a dose for a single treatment of an adult patient, which may be proportionally adjusted for children and infants, and also adjusted for other antibody formats in proportion to molecular weight and pharmacokinetics. Treatments may be by continuous infusion or may be repeated at daily, twice-weekly, weekly or monthly intervals, as appropriate. Treatment may be repeated within the same day, e.g. for treatment of short-term problems such as epilepsy and traumatic brain injury.

It is presently preferred that a whole antibody of the IgG4 subclass is used for systemic and local applications but for local applications a scFv antibody may be particularly valuable.

Specific binding members of the present invention will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the specific binding member.

Thus pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active ingredient, a

pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection solution, Ringer's Injection solution, or Lactated Ringer's Injection solution. Preservatives, stabilisers,

buffers, antioxidants and/or other additives may be included, as required.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. Other treatments may include the administration of suitable doses of pain relief drugs or anti-emetics.

The present invention provides a method comprising causing or allowing binding of a specific binding member as provided herein to an antigen. As noted, such binding may take place *in vivo*, e.g. following administration of a specific binding member, or nucleic acid encoding a specific binding member, or it may take place *in vitro*.

The amount of binding of specific binding member to an antigen may be determined. Quantitation may be related to the amount of the antigen in a test sample, which may be of diagnostic interest.

The reactivities of antibodies on a sample may be determined by any appropriate means. Radioimmunoassay (RIA) is one possibility. Radioactive labelled antigen is mixed with unlabelled antigen (the test sample) and allowed to bind to the antibody. Bound antigen is physically separated from

unbound antigen and the amount of radioactive antigen bound to the antibody determined. The more antigen there is in the test sample the less radioactive antigen will bind to the antibody. A competitive binding assay may also be used with non-radioactive antigen, using antigen or an analogue linked to a reporter molecule. The reporter molecule may be a fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The signals generated by individual antibody-reporter conjugates may be used to derive quantifiable absolute or relative data of the relevant antibody binding in samples (normal and test).

The present invention also provides the use of a specific binding member as above for measuring antigen levels in a competition assay, that is to say a method of measuring the level of antigen in a sample by employing a specific binding member as provided by the present invention in a competition assay. This may be where the physical separation of bound from unbound antigen is not required. Linking a reporter molecule to the specific binding member so that a physical or optical change occurs on binding is one possibility. The reporter molecule may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

The present invention also provides for measuring levels of antigen directly, by employing a specific binding member according to the invention for example in a biosensor system.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

The present invention further extends to a specific binding member which competes for binding to an antigen with any specific binding member which both binds the antigen and comprises a V domain including a CDR with amino acid substantially as set out herein or a V domain with amino acid sequence substantially as set out herein. Competition between binding members may be assayed easily *in vitro*, for example by tagging a specific reporter molecule to one binding member which can be detected in the presence of other untagged binding member(s), to enable identification of specific binding members which bind the same epitope or an overlapping epitope. Competition may be determined for example using the ELISA, e.g. a whole cell ELISA using immobilised brain endothelial cells, looking for inhibition of signal when cells are pre-incubated with a potential competitor antibody.

In testing for competition a peptide fragment of the antigen may be employed, especially a peptide including an epitope of interest. A peptide may have the epitope sequence plus one or more amino acids at either end, may be used. Such a peptide

may be said to "consist essentially" of the specified sequence. Specific binding members according to the present invention may be such that their binding for antigen is inhibited by a peptide with or including the sequence given. In testing for this, a peptide with either sequence plus one or more amino acids may be used.

Specific binding members which bind a specific peptide may be isolated for example from a phage display library by panning with the peptide(s).

The present invention further provides an isolated nucleic acid encoding a specific binding member of the present invention. Nucleic acid includes DNA and RNA. In a preferred aspect, the present invention provides a nucleic acid which codes for a CDR or VH or VL domain of the invention as defined above.

The present invention also provides constructs in the form of plasmids, vectors, transcription or expression cassettes which comprise least one polynucleotide as above.

The present invention also provides a recombinant host cell which comprises one or more constructs as above. A nucleic acid encoding any CDR, VH or VL domain, or specific binding member as provided itself forms an aspect of the present

invention, as does a method of production of the encoded product, which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression a VH or VL domain, or specific binding member may be isolated and/or purified using any suitable technique, then used as appropriate.

Specific binding members, VH and/or VL domains, and encoding nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes origin other than the sequence encoding a polypeptide with the required function. Nucleic acid according to the present invention may comprise DNA or RNA and may be wholly or partially synthetic. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host

cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is *E. coli*. A suitable host where a coding sequence includes a TAG suppressible stop codon, is a suppressor strain such as the sup44 strain of TG1. In such a strain, TAG codons are read as encoding glutamine. Embodiments of the present invention where a suppressor strain may be used in production of the encoded polypeptide by expression from encoding nucleic acid comprising a TAG codon, include where the coding sequence of any of SEQ ID NO's 37, 43 and 69 are used (respectively encoding G85 VH SEQ ID NO. 38, G88 VL SEQ ID NO. 44, and G112 VH SEQ ID NO. 70).

The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, A. Bio/Technology **9**: 545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a specific binding member, see for recent reviews, for example Ref, M.E. (1993) Curr. Opinion Biotech. **4**: 573-576; Trill J.J. et al. (1995) Curr. Opinion Biotech **6**: 553-560.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For

bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.
In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

The present invention also provides a method which comprises using a construct as stated above in an expression system in order to express a specific binding member or polypeptide as above.

Aspects and embodiments of the present invention will now be illustrated by way of example with reference to the following experimentation.

ABBREVIATIONS

Immunocytochemistry (ICC)

Interleukin 1-β (IL-1β)

Enzyme linked immunosorbent assay (ELISA)

Serum amyloid protein (SAP)

Bovine serum albumin (BSA)

Blood brain barrier (BBB)

LIST OF EXAMPLES

EXAMPLE 1: Generation of *in vitro* blood brain barrier cell culture chambers.

EXAMPLE 2: Selection of antibody-expressing phage which cross *in vitro* blood brain barriers.

EXAMPLE 3: Characterisation of selected antibodies by endothelial cell ELISA and sequencing.

EXAMPLE 4: Demonstration of transport of clonal phage.

EXAMPLE 5: Rat brain immunocytochemistry of endothelial-cell binding antibody clones.

EXAMPLE 6: Examination of ICC cross-reactivity of rat endothelial cell-binding antibodies with a panel of human tissues.

EXAMPLE 7: Selection of SAP-binding antibodies.

EXAMPLE 8: Characterisation of SAP-binding antibodies on the basis of sequence and ability to cross a blood brain barrier in the presence or absence of SAP.

EXAMPLE 1 - GENERATION OF IN VITRO BLOOD BRAIN BARRIER CELL CULTURE CHAMBERS

(a) Production of primary cell cultures to generate *in vitro* blood brain barrier

(i) Preparation of cultures of astrocytes from rat cerebral tissue

The protocol used for the preparation of separate cultures of astrocytes was an adaptation of the method described by K.D. McCarthy and J. De Vellis (J. Cell. Biol., 1980, vol. 85, 890-902). Using similar methods, astroglial cultures obtained are reported to appear to be >98% pure by immunocytochemistry using an antibody to glial fibrillary acidic protein (GFAP). A summary of this protocol for astrocyte cell culture has been published (G.M.A. Wells., 1996, in Glia, vol. 18, p. 332 340). The use of 12 to 24 hour old rat pups ensured the absence of viable neurons in the cell suspension obtained from dissociating the cerebral cortex and the ready dissociation of cells by mechanical sieving techniques rather than using trypsinisation. The length of the initial culture period was

kept to between 7 and 9 days to ensure stratification of the astrocytes, microglia and oligodendrocytes. The procedure for the separation of individual glial cell cultures relies upon the selective detachment of other cells from the layer of firmly attached type 1 astrocytes when the primary cell culture is exposed to sheer forces on shaking the cultures in an orbital incubator overnight (approx. 16 hours) at 37°C. The separated astroglial cell cultures can be maintained for several weeks enabling investigation of this cell type.

Fifteen 12-24 hour old rat pups were placed in a chamber containing halothane for approximately 5 minutes, or until there was no movement from the animals. The neck of each animal was broken by pinching firmly below the head. 3-4 of the rat pups were decapitated and the heads were placed upright on a pad of sterile filter paper so that blood from the cut was absorbed. A head was dabbed on the filter paper to remove excess blood, then turned upside down and the skin at the base of the neck was grasped with fingers. Skin from the back of the neck was cut to just above the eyes using dissection scissors, and the skin folded away from the skull. Both sides of the skull were then cut from the back of the neck to just above the eyes and peeled away with forceps. The optic nerves were detached with a spatula, and the cerebral hemispheres removed (without the cerebellum) from the cavity with the same spatula and placed in a sterile 50 ml tube

containing 20 ml DMEM (containing 4.5 g/l glucose and 110 mg/l sodium pyruvate - from Life Technologies) + L glutamine (Life Technologies) + 20% foetal bovine serum (FBS) (Life Technologies) + penicillin / streptomycin (Life Technologies).

The cerebral tissue in DMEM was poured from the 50 ml tube into a sterile petri dish and each individual brain tissue was removed with forceps onto a pad of sterile filter paper. The cerebral hemispheres of each brain were parted and individually rolled across the length of a fresh filter paper to remove the meninges and blood vessels. These were then placed in another petri dish containing 20 ml of fresh DMEM containing 20% FBS. The brain tissue was chopped into very small pieces using a single edged razor blade whilst immersed in the DMEM in the petri dish. A 230μm re-usable metal sieve in its cup shaped holder was placed on the top of a Sterilin pot and 20 ml of fresh DMEM containing 20% FBS was pipetted through it. The 20 ml of DMEM containing the chopped brain tissue was then taken up from the petri dish in a 25 ml pipette and pipetted onto the sieve. The chopped tissue was forced through the sieve using the glass tissue. The filtered cell suspension was divided equally between two 50 ml centrifuge tubes and centrifuged for 10 minutes at 1000 rpm. Supernatants were removed and the cell pellets resuspended by gentle pipetting in a final volume of 20 ml DMEM containing 20% FBS.

The cell suspension was then passed through a 100 μm disposable sieve placed on top of a 50 ml tube and washed through with 10 ml DMEM containing 20% FBS. The filtered cell suspension was then passed through a 70 μm disposable sieve placed on top of a fresh 50 ml tube and washed through with 10 ml DMEM containing 20% FBS. The resulting filtered cell suspension was pipetted into a 75 cm tissue culture flask and enough DMEM containing 20% FBS added so that there was a final volume of 20 ml per brain used. The cell suspension was mixed and divided into the relevant number of 75 cm flasks so that each flask contained 20 ml of cell suspension. The top of each flask was loosened and the flasks placed in a 37°C incubator with a 5% CO atmosphere.

The flasks were left undisturbed for 4 days and then inspected. The old medium and tissue debris was removed, the cells fed with 20 ml of fresh DMEM containing 20% FBS and left for a further 3 days in the incubator. On day 7 of the primary culture flasks were checked for good coverage of total cells (by this stage the astrocytes should be approaching confluency). The medium was removed and replaced with 15 ml of DMEM containing 10% FBS. The flasks were returned to the incubator with loosened tops and left for a further 24-48 hours. Astrocytes were passaged, with a 1:4 dilution of cells, approximately every week (or upon reaching confluency) over a

period of several weeks using DMEM containing 10% FBS as the standard growth medium.

(ii) Preparation of cultures of rat brain endothelial cells. Attempts to establish highly enriched cultures of brain endothelial cells (EC) from several species have resulted in a variety of methods based on disruption of cerebral tissue (usually by a combination of mechanical and enzymatic means), followed by purification of microvessels by density centrifugation, before plating out on tissue culture plastic coated with extracellular matrix protein (for review see F. Joo, 1992, in J. Neurochem, vol. 58, p. 1 17). The protocol was adapted from the published methods of C. C. W. Hughes and P. L. Lantos for the establishment of EC cultures from microvessels derived from rat cerebral tissue. The use of only dissected out cerebral grey matter reduces the amount of myelin in the enzymatic digest of the tissue. Tissue dissected from two Wistar rats results, digested and purified using this protocol results in a primary cultures of microvessels set up in two 6 well dishes from which individual colonies of endothelial cells can be trypsinised out after 7 days in culture.

The cerebral tissue in DMEM was poured into a sterile petri dish and each piece of tissue was removed onto a pad of sterile filter paper. The cerebral hemispheres were parted and

the meninges and large blood vessels were removed by peeling off the outer layer of the tissue and rolling the tissue along the length of the filter paper pad. The grey matter was dissected away from the white matter, pooled together and placed into a fresh petri dish containing sterile serum free DMEM containing penicillin and streptomycin. This was then chopped up into fine pieces using a single sided razor blade. The chopped up tissue was then placed into a sterile 50 ml centrifuge tube and pelleted at 200 g for 10 minutes at room temperature. The supernatant was removed and the tissue was resuspended in 10 ml of serum free DMEM containing penicillin and streptomycin plus 1 mg/ml collagen (Boehringer Mannheim, cat. no. 269 638) and 10 μg/ml DNase I (Sigma Type II, cat. no. D4263). This was then placed on a shaking incubator at 37°C for 90 minutes. The tissue digest was then pelleted at 200 g for 10 minutes at room temperature and resuspended in 40 ml of DMEM containing 25% (w/v) bovine serum albumin (BSA, Fraction V from Sigma, cat. no. A9647) with trituration until it had a creamy texture. This was then spun at 1000 g for 20 minutes at room temperature to pellet the capillary fragments (heavier) from myelin, astrocytes, neurons and other single cell contaminants (lighter). The upper layer was carefully removed making sure to remove all traces of the myelin etc. The capillary pellet was resuspended in 10 ml serum free DMEM and re-pelleted at 600 minutes at room temperature. The resulting capillary pellet was then resuspended in 10 ml DMEM

containing penicillin and streptomycin plus 1 mg/ml collagenase/disease and incubated at 37°C for 3 hours with shaking. The digest was then pelleted at 600 g for 5 minutes at room temperature and resuspended with trituration in 10 ml of endothelial cell growth medium (ECGM) which consists of DMEM containing 4.5 g/l glucose and 110 mg/ml sodium pyruvate (Sigma) supplemented with 2 mM L-glutamine (Sigma), 100 units/ml penicillin plus 100 μg/ml streptomycin (Sigma), 20% plasma derived foetal calf serum (First Link (UK) Ltd., cat no. 60 00 850) and 75 μg/ml endothelial cell growth supplement (ECGS Sigma, cat. no. E 2759). The suspension was then passed through successive 230, 100 and 40 μm sterile sieves with gentle, washing of each sieve with 10 ml of fresh ECGM. The resulting sieved suspension was plated out into 6 well plates (3 4 ml per well) coated with type 1 collagen from rat tail (Becton Dickinson, cat. no. 40236). The 6 well plates and all flasks used below were coated with 100 μg/ml type 1 collagen (diluted from stock concentration in sterile 0.02N acetic acid) for 1 hour at room temperature with gentle rocking, before removing the collagen and rinsing with PBS. Enough diluted collagen was added to cover the entire surface area of the tissue culture plastic (>5 $\mu g/cm^2$). This gave rise to the primary enriched microvessel cultures from which endothelial cells were further enriched from potential contaminants as described below.

The medium was replaced on the primary cultures with fresh ECGM after 4 days. Large colonies of cells with endothelial like morphology were observed after 7 days in culture having grown out radially in spirals from pieces of microvessel attached to the tissue culture plastic. Over a period of several days, colonies of cells, which were thought to be endothelial cells, were transferred aseptically into fresh 6 well plates (coated with collagen type 1) using small pieces of filter paper soaked in trypsin/EDTA. After a brief period in contact with the filter paper, the trypsinised EC stick to the filter paper after detachment from the well and can be transferred to another plate. Once these transferred cells form colonies which had expanded to cover the entire surface area of the 6 well dish over a period of 3-4 days (passage 1 cells), wells were trypsinised and placed into collagen coated T_{25} flasks (passage 2), cultured for a further 3-4 days and then passaged into collagen coated T_{75} flasks (passage 3). Once the T75 flasks had reached confluency they were either frozen down in liquid nitrogen or expanded with two 1:5 passages to yield a large stock of flasks for preparation of liquid nitrogen stocks.

(b) Preparation of *in vitro* blood brain model in tissue culture inserts

It has been found that brain ECs can be cultured successfully on permeable filters that separate an upper from a lower chamber in the commercially available tissue culture inserts. As these are available in multi-well formats numerous conditions can be tested simultaneously to determine the integrity of the barrier or investigate specific transport mechanisms. The electrical resistance measured across a monolayer of cells that are connected to each other by tight junctions is a sensitive indicator for the paracellular permeability to ions. Thus the quality of the EC barriers can be simply recorded to provide assurance for the model. It is thought that type 1 astrocytes, which normally project to brain capillary ECs, are able to provide some inductive influences in making ECs less leaky by inducing the formation of tight junctions of extremely high electrical resistance. Various combinations of astrocytes and endothelial cells have been tried in establishing in vitro models of the BBB. For good reproducible results the culture of ECs in the upper chamber on the permeable membranes and astrocytes in the lower chamber on the base of the companion 24 well plate has been demonstrated to yield barriers with a high transendothelial cell resistance. This was the arrangement of cells used as described in the protocol below.

Cultures of rat astrocytes and endothelial cells (EC) were set up set up several days/weeks in advance from frozen stocks

stored in liquid nitrogen. These were kept at as low passage number as possible to preserve the normal characteristics of the primary cells (astrocytes at less than passage 6, and endothelial cells at less than passage 10). After thawing a vial from liquid nitrogen storage, astrocytes were cultured in astrocyte growth medium (AGM) consisting of DMEM containing 4.5 g/l glucose and 110 mg/l sodium pyruvate (Life Technologies) supplemented with 2mM L-glutamine (Sigma), 10% foetal bovine serum (FBS)(Life Technologies), 100 units/ml penicillin plus 100 µg/ml streptomycin (Sigma). Likewise, endothelial cells were cultured in endothelial cell growth medium (ECGM) described in section B consisting of DMEM containing 4.5 g/l glucose and 110 mg/ml sodium pyruvate (Sigma) supplemented with 2 mM L-glutamine (Sigma), 100 units/ml penicillin plus 100 µg/ml streptomycin (Sigma), 20% plasma derived foetal calf serum (First Link (UK) Ltd., cat no. 60-00-850) and 75 µg/ml endothelial cell growth supplement (ECGS-Sigma, cat. no. E 2759).

Initially in setting up the barriers in the tissue cultures, astrocytes were placed in the lower chamber and allowed to adhere before placing the tissue culture insert into the plate and adding the endothelial cells to the upper chamber. A T_{175} flask of astrocytes was trypsinised and the number of cells counted in a haemocytometer. These were then diluted to 1.0 x 10^5 cells/ml in AGM and 1 ml of cell suspension was added to

the wells of the 24 well lower plate. The astrocytes were allowed to adhere to the surface of the lower chamber for at least 2 hours in a CO_2 incubator. While the astrocytes were settling down, the appropriate number of 8 μm inserts were coated with 100 μg/ml rat tail type 1 collagen before adding the endothelial cells. The inserts were placed in a separate lower plate and 1 ml of diluted collagen was added to the lower chamber and 0.5 ml of collagen was added to the upper chamber. The inserts were then left for at least 1 hour at room temperature in a class II tissue culture cabinet. The collagen was then removed and the inserts were rinsed in PBS. Once the inserts had been coated with collagen and rinsed with PBS, a T_{175} flask of EC was trypsinised and the number of cells counted in a haemocytometer. These were then diluted to 2.0×10^5 cells/ml in AGM. The lower plate containing the cultures of astrocytes was removed from the incubator and the medium on the astrocytes was replaced with 1 ml of fresh AGM. The coated inserts were then placed in the lower plate containing the astrocyte cultures using forceps sterilised in 70% ethanol and 0.5ml of the 2.0×10^5EC/ml suspension was added to the upper chamber. The plates were placed back in the CO_2 incubator and not disturbed for at least 24 hours.

The next day the barrier cultures were inspected under the microscope. The EC should have formed a near confluent coating of the upper chamber, and, likewise, the astrocytes in

the lower chamber should have spread out over most of the surface area of the lower chamber. At this stage the electrical resistance of EC barrier can be measured, however, it takes several days before the resistance rises as the cells need to reach confluency and then form tight junctions. The medium in both the upper and lower chambers was replaced with fresh AGM every 7 days and electrical resistance monitored with time. High levels of electrical resistance are obtained after at least 2 weeks in culture. These cultures have been maintained for up to 8 weeks before any fall in electrical resistance is observed.

(c) Measurement of electrical resistance of endothelial cell barrier

Measurement of electrical resistance across the tissue culture insert membrane can be performed under sterile conditions to monitor the progress of the endothelial cell cultures in forming a good model of the blood brain barrier in the inserts. The use of the Endohm chamber electrodes for the 24 well inserts with its concentric electrodes situated above and beneath the membrane reduces the background resistance to between 20 and 30 Ω for an empty 8 μm tissue culture insert. This background figure was measured on each occasion and subtracted from actual readings obtained with inserts containing EC. Before measuring any resistances, the Endohm

electrode was sterilised using 70% ethanol. The cup shaped electrode was filled with 70% ethanol and the upper electrode placed back in place. The ethanol was then tipped out and the electrode drained. To remove any final traces of the 70% ethanol the electrode was then rinsed in sterile astrocyte growth medium (AGM) a couple of times. 1 ml of fresh AGM was added to the lower chamber of the electrode and the electrode was ready for measuring the resistances of any inserts. The resistance of an empty insert was measured at the same time as the inserts containing EC and this was subtracted from all the barrier insert resistances. To obtain standardised resistance measurements, the resistance values recorded on the volt/ohm meter were divided by the surface area of the membrane (for the 24 well sized inserts used here this was 0.3cm) to give values measured in ohms/cm^2. Resistances were generally in the 500-600 ohm/cm^2 range.

EXAMPLE 2 - SELECTION OF ANTIBODY-EXPRESSING PHAGE WHICH CROSS IN VITRO BLOOD BRAIN BARRIERS

Antibody repertoire

A large single chain Fv library derived from lymphoid tissues including tonsil, bone marrow and peripheral blood lymphocytes was used.

Polyadenylated RNA was prepared from the B-cells of various lymphoid tissues of 43 non-immunised donors using the

"Quickprep mRNA Kit" (Pharmacia). First-strand cDNA was synthesized from mRNA using a "First-strand cDNA synthesis" kit (Pharmacia) using random hexamers to prime synthesis. V-genes were amplified using family-specific primers for VH, Vλ and Vκ genes as previously described (Marks et al., (1991) J. Mol. Biol. **222**:581-597) and subsequently recombined together with the $(Gly_4, Ser)_3$scFv linker by PCR assembly. The VH-linker-VL antibody constructs were cloned into the *Sfi* I and *Not* I sites of the phagemid vector, pCantab6. Ligation, electroporation and plating out of the cells was as described previously (Marks et al, supra). The library was made ca. 1000x larger than that described previously by bulking up the amounts of vector and insert used and by performing multiple electroporations. This generated a scFv repertoire that was calculated to have ca. 1.3×10^{10} individual recombinants which by BstNI fingerprinting were shown to be extremely diverse.

Induction of phage antibody library

The phage antibody repertoire above was selected for antibodies which cross the blood brain barrier.

The scFv repertoire was treated as follows in order to rescue phagemid particles. 500 ml prewarmed (37 ºC) 2YTAG (2YT media supplemented with 100 µg/ml ampicillin and 2% glucose) in a 2l conical flask was inoculated with approximately 3×10^{10} cells from a glycerol stock (-70ºC) culture of the library. The culture was grown at 37ºC with good aeration until the OD_{600nm}

reached 0.7 (approximately 2 hours). M13K07 helper phage (Stratagene) was added to the culture to a multiplicity of infection (moi) of approximately 10 (assuming that an $_{600nm}$ of 1 is equivalent to 5 x 10^8 cells per ml of culture). The culture was incubated stationary at 37ºC for 15 minutes followed by 45 minutes with light aeration (200 rpm) at the same temperature. The culture was centrifuged and the supernatant drained from the cell pellet. The cells were resuspended in 500 ml 2YTAK (2YT media supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin), and the culture incubated overnight at 30ºC with good aeration (300 rpm).

Phage particles were purified and concentrated by one polyethylene glycol (PEG) precipitation (Sambrook, J., Fritsch, E.F., & Maniatis, T. (1990) *Molecular Cloning - A Laboratory Manual* Cold Spring Harbor, New York) and resuspended in 9ml 10mM Tris containing 1 mM EDTA (TE). 4.0g of CsCl was added to the phage stock and mixed gently to dissolve. A 11.5ml ultracentrifuge tube was filled with phage at centrifuged at 40 000 rpm at 25ºC for 24 hr. The ultracentrifuge was stopped with the brake off and the clear opalescent phage band collected using a pasteur pipette. Phage were dialysed at 4ºC overnight against two changes of 1l of TE, titred and stored at 4ºC.

Selection of phage from the large phage library which cross in

vitro blood brain barriers set up in chamber slides.

The *in vitro* BBB chambers provide a valuable system which can be utilised as a means to functionally select for phage antibodies which cross the *in vitro* barriers. The phage antibody library can be placed in the top chamber of the *in vitro* barrier and phage which are capable of passing through the barrier and reaching the bottom reservoir can then be collected, and the process repeated. This is the basis on which the following selections were carried out.

(i) First round of selection

1 x 10^{12} library phage, or control phage which do not bind rat endothelial cells, in 50 μl TE were added to top reservoir of endothelial culture chambers containing rat brain endothelial cells growing as a monolayer in the presence of astrocytes as described in Example 1. Chambers were incubated at either 37ºC in a CO_2 incubator, or on ice on the bench. Media present in the lower reservoir was collected and replaced with fresh media at the appropriate temperature 30, 60 and 120 minutes after addition of the library phage to the top chamber (wells 1a, b, c and 7a, b, c). For other chambers transport was allowed to continue for 30, 60 or 120 minutes and media collected only at the final time point. The number of phage present in the media in the lower chamber (i.e. the number of transported phage) was titred. Titring was achieved by adding 0.5 ml of the media recovered from the bottom chamber (out of

a total of 1ml) to a 5ml culture of exponentially growing *E. coli* TG1 with light aeration in 2TY broth at 37ºC for 1 hour. Infected TG1's were plated on 2TYAG medium in 243mm x 243mm dishes. Dilutions of infected TG1s were also plated out and incubated at 30ºC overnight. Colony counts gave the phage output titre.

Results of titration experiments from the first round of selection are shown in Table 1.

Average initial resistance 606 Ωcm^2.

The phage population from well 7a was chosen as the input population for a second round of selection.

(ii) Second round of selection

Colonies were scraped off the 7a 243mm x 243mm plate into 3 ml of 2TY broth and 15% (v/v) glycerol added for storage at -70ºC. Glycerol stock solutions from the first round of selection were rescued using helper phage to derive phagemid particles for the second round of selection. 250 µl of the 7a glycerol stock was used to inoculate 50 ml 2YTAG broth, and incubated in a 250 ml conical flask at 37ºC with good aeration until the OD_{600} nM reached 0.7 (approximately 2 hours). M13K07 helper phage (moi=10) was added to the culture which was then incubated stationary at 37ºC for 15 minutes followed by 45

minutes with light aeration (200rpm) at the same temperature. The culture was centrifuged and the supernatant drained from the cell pellet. The cells were resuspended in 50 ml prewarmed 2YTAK, and the culture incubated overnight at 30°C with good aeration. Phage particles were purified and concentrated by PEG precipitation (Sambrook et al., 1990) and resuspended in TE to 10^{13} tu/ml, then caesium banded as described in Example 2.

Library phage, pCantab6 phage (which is identical library except it has only myc and his tags fused with the gene III protein and no scFv) and the second round 7a phage were added to top reservoirs of *in vitro* blood brain barrier culture chambers using $1x10^{12}$ phage per chamber in 50 µl TE. Chambers were incubated at 37°C in a CO_2 incubator. The lower reservoir was removed at 30 min and 500 µl of this used to replace the top reservoir of a fresh barrier culture. After a further 30 min the lower reservoir of this barrier culture was removed and 500 µl of the media used to replace the top reservoir of a fresh barrier culture. This was repeated one final time after a further 30 min.

Colonies were plated out and titred as described above, and the results of the titration experiments from the second round of selection are shown in Table 2.

Population 24 was chosen as the starting population for a third round of selection.

(iii) Third round selection

Population 24 from the second round selection regime was rescued and caesium banded as described above for the 7a second round population. Library phage, pCANTAB6 phage, population 7a and population 24 phage were added to the top reservoir of precooled barrier cultures using 1 x 10^{12} phage per chamber in 50 µl TE. Chambers were incubated for 30 min on ice and washed 3 times with 0.5 ml of ice cold media. Insert and liquid within the upper chamber were then transferred to a fresh chamber well containing 1 ml of media at 37ºC and the chambers incubated at 37ºC in a CO_2 incubator. The lower reservoir was removed at 30 min and 500 µl of this used to replace the top reservoir of a fresh barrier culture. After a further 30 min the lower reservoir of this barrier culture was removed and 500 µl of the media used to replace the top reservoir of a fresh barrier culture. This was repeated one final time after a further 30 min and the barrier culture incubated for 30 min at 37ºC.

Colonies were plated out and titred as described above (Table 3).

Growth of single selected clones for immunoassay

Individual colonies from the third round selection (chambers 25, 26 and 29) were used to inoculate 100 µl2YTAG into individual wells of 96 well tissue culture plates (Corning). Plates were incubated at 30°C overnight with moderate shaking (200 rpm). Glycerol to 15 % was added to each well and these master plates stored at -70°C until ready for analysis.

EXAMPLE 3 - CHARACTERISATION OF SELECTED ANTIBODIES BY ENDOTHELIAL CELL ELISA AND SEQUENCING

Endothelial cell phage ELISA

Selected phage were analysed by phage ELISA for their ability to bind to culture rat endothelial cells. Phage ELISAs were carried out as follows: individual clones were picked into a 96 well tissue culture plate containing 100 µl 2YTAG. Plates were incubated at 37°C for 6 hours. M13KO7 helper phage was added to each well to an moi of 10 and incubated with gentle shaking for 45 min at 37°C. The plates were centrifuged at 2000 rpm for 10 min and the supernatant removed. Cell pellets were resuspended in 100 µl 2TYA with kanamycin (50 µg/ml) and incubated at 30°C overnight. Each plate was centrifuged at 2000 rpm and the 100 µl phage-containing supernatant from each well recovered and blocked in 20 µl 6x PBS containing 18% marvel stationary at room temperature for 1 hour. Meanwhile, 96 well tissue culture plates, containing either 1 x 10^5 endothelial

cells per well or control uncoated plates, were blocked for 2 h stationary at room temperature in PBS containing 3% Marvel (3MPBS). These plates were then washed three times with PBS and 50 µl preblocked phage added to each well. The plates were incubated stationary at room temperature for 1 h after which the phage solutions were poured off. The plates were washed by incubating for 2 min in three changes of PBS at room temperature.

To the ELISA plate well, 100 µl of a 1 in 5000 dilution of the anti-gene8-HRP conjugate (Pharmacia) in 3MPBS was added and the plates incubated at room temperature stationary for 1 h. Each plate was washed as described. 100 µl of TMB substrate was then added to each well, and incubated at room temperature for approximately 30 minutes, after which the colour reaction was stopped by the addition of 50 µl of 1M H_2SO_4. The absorbance signal generated by each clone was assessed by measuring the optical density at 405 nm using a microtitre plate reader. Clones were chosen for further analysis if the ELISA signal generated on the cell-coated plate was at least double that of the uncoated plate. Of 96 clones screened from the third round of selection 20 were positive by endothelial cell ELISA.

Sequencing of anti-endothelial cell ScFv Antibodies

The nucleotide sequences of the rat endothelial cell binding

antibodies were determined by first using vector-specific primers to amplify the inserted DNA from each clone. Cells from an individual colony on a 2YTAG agar plate were used as the template for a polymerase chain reaction (PCR) amplification of the inserted DNA using the primers pUC19reverse and fdtetseq. Amplification conditions consisted of 30 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2min, followed by 10 min at 72ºC. The PCR products were purified using a PCR Clean-up Kit (Promega) in to a final volume of 50 µlH_20. Between 2 and 5 µl of each insert preparation was used as the template for sequencing using the Taq Dye-terminator cycle sequencing system (Applied Biosystems). The primers pUC19reverse and fdtseq were used to sequence the heavy and light chain of each clone respectively. The diversity of the CDR3s and the germline families is shown in Table 4. Detailed sequence data is included below.

EXAMPLE 4 - DEMONSTRATION OF TRANSPORT OF CLONAL PHAGE.

Four phage antibody clones, that gave positive endothelial cell ELISA results and gave endothelial specific staining by ICC on rat brain, were tested for their ability to cross the *in vitro* blood brain barrier as clonal preparations. Phage were prepared by rescue and caesium banding as described Example 2. $1x10^8$ phage in 50 µl TE were added to the upper reservoir of *in vitro* blood brain barrier chamber cultures

which had been pre-chilled on ice. The phage were allowed to bind to the endothelial cell layer for 30 min on ice and the cell layer then washed 3 x with 1ml ice cold media. The chamber inserts were transferred to media prewarmed to 37ºC and incubated at 37ºC in a CO_2 incubator to allow transport to proceed. Phage crossing the barrier were titred, as described above, following the 30 min on ice then following 30 at 37ºC (Table 5).

In the cases of the four test clones (G65, G73, G77 and G93) the number of phage actively crossing the barrier after 30 minutes at 37ºC compared with the number of phage crossing non-specifically at 4ºC was greatly increased. G65 showed a 12-fold increase over background transport (No. phage crossing at 37ºC / No. of phage crossing at 4ºC), G73 a 2.6-fold increase, G77 a 12.8 fold increase and G93 a 4.3-fold increase. The control phage pCantab6 which expresses no surface scFv showed no increase in transport at 37ºC over transport at 4ºC.

EXAMPLE 5 - RAT BRAIN IMMUNOCYTOCHEMISTRY OF ENDOTHELIAL-CELL BINDING ANTIBODY CLONES

The twenty clones identified by cell ELISA as binding to activated rat endothelial cells were also tested by immunocytochemistry (ICC) for their ability to bind to

inflamed rat brain sections.

Preparation of rat brain sections.

3 week-old Lewis rats were anaesthetised with avertin (1ml / 100g) and placed in a sterotaxic frame. Injections of 1µl of interleukin-1β (10 units) were placed into, the striatum. An incision was made in the scalp to expose bone and a 2-mm diameter burr hole was drilled through the skull to allow the tip of a finely drawn calibrated glass capillary tube to be inserted. Rats were deeply anaesthetised with sodium pentobarbitone and transcardialy perfused with 100ml of saline followed by 200ml of Karnovsky's fixative (1.25% gluteraldehyde and 1.25% paraformaldehyde in phosphate buffer). The animals were recovered from the anaesthetic before being killed. The brain was removed and cryoprotected in 30% sucrose overnight at 4ºC before being embedded in Tissue-Tek (Miles Inc, Elkhart, USA) and quickly frozen in liquid nitrogen. 5 micron cryosections were taken of treated brains.

Preparation of phage for ICC

Phage clones were inoculated into 1ml 2TYGA in a deep well microtitre plate and grown at 37ºC with aeration for 5 hours. M13K07 helper phage was added to each well at an moi of 10 and incubated with gentle shaking for 45 min at 37ºC. The plates

were centrifuged at 2000 rpm for 10 min and the supernatant removed. Cell pellets were resuspended in 1ml 2TYKA and incubated at 25ºC overnight. Plates were centrifuged at 2000 rpm for 10 min and the phage supernatant collected from each well ready for use in ICC.

ICC protocol

Rat brain sections were fixed by immersion in acetone at ambient temperature for 15 min, and washed twice for 3 min in PBST. Sections were blocked in 5 µg/ml streptavidin in PBST for 15 min, washed 3 times 3 min in PBST and incubated in 10 µg/ml biotin in PBST for 15 min. Sections were washed 5 times 3 min in PBST, then twice for 10 min in wash containing 1% BSA. 1% BSA was added to the phage supernatants and phage incubated on the sections for 2 hr at ambient temperature. Slides were washed 5 times for 3 min in PBST and incubated with an anti-M13-HRP conjugate (Pharmacia) diluted 1/500 in PBST containing BSA. Sections were washed 5 times 3 min in PBST and a biotin tyramine amplification step then carried out. Biotin tyramine amplification consisted of incubation of the section with biotin tyramine diluted 1/600 in 50mM Tris-HCl, pH 7.4 containing 0.03% hydrogen peroxide for 10 min at room temperature, after which the slides was washed in twice for 3 min in PBST. Sections were then incubated for 30 min in SABC-HRP complex (DAKO KO377) diluted in PBST, and then washed 5 times for 3 min in PBST. Sections were stained by

incubation with 3-amino-9-ethyl-carbazole (AEC, Sigma) at a concentration of 2.4 mg/ml in dimethylformamide. ACE incubation was for 3 min followed by washing in 0.1% tween, and this was repeated twice. Slides were further washed in 0.1% tween twice for 5 min and the slides then counterstaining with haemotoxylin (DAKO) for 10 sec. Seven changes of 3 min washing in water were then carried out and the sections coated in aqueous mount.

Summary of results.

18 of the 20 ELISA positive clones gave staining of vessel walls in rat brain sections. Profiles varied in intensity of staining. No staining was observed in tissues other than vessels. Control sections in which phage antibody was omitted gave no specific staining of the rat brains.

EXAMPLE 6 - EXAMINATION OF ICC CROSS-REACTIVITY OF RAT ENDOTHELIAL-BINDING PHAGE ANTIBODY CLONES ON A PANEL OF HUMAN TISSUES

The rat endothelial cell clones positive by ICC on rat brain were tested on a range of normal and diseased human tissues for cross-reactivity.

Preparation of phage and ICC.

Phage were prepared for ICC as described in Example 5. The

panel of human tissues prepared for ICC consisted of:

Normal tissues	Diseased tissues
Tonsil	Adenocarcinoma of breast
Kidney	Cirrhosis of liver
Cerebellum	Lung carcinoma
Spinal cord	Crohn's disease
Peripheral nerve	Ulcerative colitis
Cerebrum	
Striated muscle	
Testis	
Skin	
Spleen	
Lung	
Breast	
Liver	
Heart	
Myometrium	

ICC results

ICC was carried out exactly as described in Example 5. Six (G65, G77, G102, G81, G101, G112) out of the 18 clones tested gave no specific staining on the panel of human tissues.

5 clones gave staining in the cerebellum: G73 was cerebellum specific; G110 stained adenocarcinoma and striated muscle in

addition to cerebellum; G95 stained smooth muscle in addition to cerebellum; G93 stained spinal cord in addition to cerebellum; G92 stained spinal cord and cerebrum in addition to cerebellum.

G76 stained peripheral nerve and cell bodies in spinal cord.

Of the remaining clones a number of other staining patterns were observed. G88 gave specific staining of lung carcinoma. G83 stained kidney glomeruli and leydig cells of the testis. G92 gave staining of the leydig cells of the testis, plus other punctate staining of the testis, and some staining of striated muscle.

ICC staining patterns are summarised in Table 6.

EXAMPLE 7 - SELECTION OF SAP-BINDING ANTIBODIES

Serum Amyloid Protein (SAP) is specifically transported across the blood brain barrier. Phage antibodies binding to human SAP (hSAP) were isolated, as outlined below, and a phage antibody clone (D5) which gave a strong signal by ELISA on hSAP was examined for its ability to be transported across an *in vitro* BBB.

Isolation of Phage binding to hSAP.

hSAP was coated onto the surface of a Maxisorp tube using 1 ml of 10µg/ml in PBS overnight at 4ºC. After coating the Maxisorp tube was rinsed three times with PBS and filled to the brim with 3% (w/v) skimmed milk powder in PBS (3MPBS) and blocked for 1h at 37ºC. The library phage were blocked at room temperature for 1h with 3MPBS. The coated Maxisorp tube was rinsed three times with PBS, and 1 ml of pre-blocked phage was added to the Maxisorp tube. Phage was incubate for 1 h at 37ºC, and the tube then rinsed twenty times with PBST, then twenty times with PBS. The bound phage were eluted by the addition of 1 ml of freshly made 100 mM triethylamine to the Maxisorp tube. The tube was incubated (stationary) at room temperature for 10 min and eluted phage were transferred to a 1.5 ml microcentrifuge tube. The phage were neutralised by the addition of 500µl 1 M Tris buffer pH 7.4. A single colony of *E. coli*. TG1, taken from a minimal agar plate, was used to inoculate 50 ml of 2TY broth and incubated with shaking at 37ºC until an $A._{600}$ of 1.0 had been achieved. 5 ml of the exponentially growing TG1 were placed in a 50 ml Falcon tube, 750µl (half) of eluted phage added and infection carried out by incubation at 37ºC for 30 min stationary and for 30 min shaking (<200 r.p.m.). The remainder of the eluted phage was stored at 4ºC as a back-up. The infected cells were centrifuged 3500 r.p.m. for 10 min and the cell pellet resuspended in 0.6 ml 2TY broth and spread on one 243 x 243 mm 2TYAG agar plate. Colonies were grown at 30ºC overnight.

The colonies were scraped into 10 ml of 2TY broth in a 50 ml Falcon tube, 5 ml of sterile 50% v/v glycerol was added and mixed by placing tube on an end-over-end rotator for 10 min at room temperature. 25 ml of 2TYG + 100μg/ml ampicillin was inoculated with around 50μl of the above plate scrape and grown at 37ºC to an A_{600} of 0.5 to 1.0. M13K07 helper phage was added to a final concentration of 5 x 10^8 pfu/ml and the cells infected at 37ºC for 30 min stationary and for 30 min shaking (<200 r.p.m.). The cells were transferred to a 50 ml Falcon tube and centrifuged at 3500 r.p.m. for 10 min then the bacterial pellet resuspended in 25 ml prewarmed 2TY with kanamycin (50μg/ml) and ampicillin (100μg/ml). This was transferred to a fresh 250 ml flask and grown overnight with rapid shaking (300 r.p.m.) at 30ºC to produce phage particles. Approximately 1 ml of culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 13000 r.p.m. in a microfuge. The phage-containing supernatant was transferred to a fresh tube and used as the input population for a second round of selection which was performed exactly as described above.

Screening of selected clones by phage ELISA on hSAP.

Individual colonies were picked into 100μl of 2TYAG in a 96 well microtitre plate and grown at 30ºC shaking at 100 rpm overnight. The cultures in this plate were used to inoculate a

fresh plate and 50µl of 50% v/v glycerol was added to the original plate and it was stored frozen at -70ºC. The fresh plate was grown at 37ºC for 5-6 hrs until the cultures were turbid. To each well of the replica plate 10µl of M13K07 in 2TYG + 100ug/ml ampicillin (at 5 x 10^{10} pfu/ml, an m.o.i. of 10) was added. The plate was incubated for 30 min without shaking at 37ºC and then for 30 min with shaking (100 r.p.m.) at 37ºC to allow superinfection of the helper phage. The cultures were pelleted at 2,000 rpm for 10 min and the supernatant discarded. The bacterial pellets were resuspended in 100µl of 2TYAK and grown overnight at 30ºC shaking at 100 rpm.

The cultures were centrifuged at 2,000 rpm for 10 min and the supernatant transferred to a fresh plate. The phage was blocked by the addition of 20µl of 6 x PBS/18% Marvel, mixed by pipetting and incubated for 1 h at room temp.

Antigen plates were prepared by adding 100µl of 10µg/ml hSAP in PBS to microtitre plate wells and incubating overnight at 4ºC. Plates were washed 3 x in PBS and blocked for 1 h in 3% MPBS at 37ºC. Phage ELISAs were performed as described in Example 3.

Approximately 30% of the phage antibody clones screened by

ELISA were found to be positive in terms of binding to hSAP compared to BSA or an uncoated ELISA plate. One clone, D5, which gave the highest ELISA signal was chosen for further transport studies. Clone D5 was sequenced (as described in Example 3) and the relevant sequences are included below (SEQ ID NO. 73 encoding VH domain SEQ ID NO. 74; SEQ ID NO. 75 encoding VL domain SEQ ID NO. 76).

EXAMPLE 8: CHARACTERISATION OF THE SAP-BINDING PHAGE ANTIBODY D5 ON THE BASIS OF ITS ABILITY TO CROSS AN IN VITRO BBB IN THE PRESENCE OR ABSENCE OF SAP

Experiments were carried out in serum free media to avoid competition between any endogenous SAP and the added hSAP. Phage, either D5 or pCANTAB6, were added to the upper reservoir of ice cold BBB cultures in the presence or absence of 10μg/ml hSAP at 10^8, 10^6, or 10^4 phage per chamber. The cultures were incubated on ice for 30 min and the liquid in the lower reservoir then removed for titring. The upper chamber was washed 3 x with 0.5 ml of ice-cold media then transferred to a fresh well and incubated at 37°C in a CO_2 incubator for 30 min. Liquid from the lower reservoir was again removed and the phage titre present determined by titration (Table 7).

Transport of the D5 phage is increased approximately two fold

in the presence of hSAP whereas the transport of pCANTAB6 is reduced slightly in the presence of hSAP. Overall the D5 phage were transported 10-fold more efficiently than the pCantba6 phage in the absence of SAP, and 100-fold more efficiently in the presence of SAP. This provides indication that the D5 phage are being actively transported, and the higher level of D5 transport in the absence of SAP may be a result of endogenous SAP which is pre-bound to the rat endothelial cells.

Table 1.

Time (min)	37°C well	phage	titre	Resistance Ω/cm^2
30	1a	library	$2.5x10^6$	
60	1b	library	$2.5x10^6$	
120	1c	library	$2.5x10^6$	310
60	2	library	$2.5x10^6$	
120	3	library	$2.5x10^6$	
30	4	Fat67	$1.5x10^6$	
60	5	Fat67	$2.5x10^6$	
120	6	Fat67	$2.5x10^6$	453
Time (min)	4°C well	phage	titre	Resistance Ω/cm^2
30	7a	library	$4x10^6$	
60	7b	library	$4x10^6$	
120	7c	library	$3x10^6$	500
60	8	library	$4x10^6$	
120	9	library	$4x10^6$	
30	10	Fat67	$1x10^6$	
60	11	Fat67	$1x10^6$	
120	12	Fat67	$2x10^6$	500

Table 2.

Well	Input phage	Output titre	Initial resistance Ω/cm^2
16	Library	$3.8x10^5$	750
17	1/2 of 16 output	20	816.7
18	1/2 of 17 output	0	670
19	1/2 of 18 output	0	573.3
20	pCANTAB6	$3x10^6$	750
21	1/2 of 20 output	0	790
22	1/2 of 21 output	0	716.7
23	1/2 of 22 output	0	716.7
24	Population 7a	$6x10^6$	690
25	1/2 of 24 output	34	790
26	1/2 of 25 output	0	530
27	1/2 of 26 output	0	N.D.

Table 3.

Well	Input phage	Time (min)	Output titre	Initial resistance Ω/cm^2	Final resistance Ω/cm^2
16	Library	30	$1.2x10^7$	603	583
17	1/2 of 16 output	30	300	647	496
18	1/2 of 17 output	30	5	647	750
19	1/2 of 18 output	30	0	613	690
20	pCANTAB6	30	$1.5x10^3$	660	570
21	1/2 of 20 output	30	0	647	633
22	1/2 of 21 output	30	0	593	706
23	1/2 of 22 output	30	0	587	720
24	Population 7a	30	$2.6x10^7$	637	643
25	1/2 of 24 output	30	$2.4x10^3$	620	660
26	1/2 of 25 output	30	8	647	753
27	1/2 of 26 output	30	0	640	750
28	Population 24	30	$5.2x10^8$	670	663
29	1/2 of 28 output	30	$1x10^4$	620	650
30	1/2 of 29 output	30	0	627	720
31	1/2 of 30 output	30	0	680	676

Table 4.

Clone	VL	VL CDR3	SEQ ID NO.	VH	VH CDR3	SEQ ID NO.
G65	DPL16	NSRDSSGNHVV	77	DP-49	TGEYSGYDTSGVEL	78
G67	DPL16	HSRDSSGNHVL	79	DP-35	DFVATMVRGAPTRVLRS	80
G73	DPL16	NSRDSSGNHVV	81	V3-30	TGEYSGYDTSGVEL	82
G76	DPL16	NSRDSSGNHVV	83	DP71	GPLRLRAFDL	84
G77	DPL8	QSYDSSLSNMI	85	DP-5	ASETRVMVDDVFNV	86
G78	DPL16	NSRDSSGNHVV	87	DP71	GPLRLRAFD	88
G79	DPL16	HSRDSSGNHVL	89	DP-7	RSGALGGLIPLNYFDY	90
G81	DPL11	NSRDSSGNHVV	91	VII-5+	VTNGHWYYFDY	92
G83	L12a+	QQSYSTPWT	93	DP-71	AASLSCTGSSCRYNYFDP	94
G85	DPK4	ENYNSVPLS	95	DP-72	GDGSDYYAMDY	96
G88	L12a+	QQYSNYPLT	97	2M27	EGTAIHRAFDI	98
G92	DPL2	ASWDDSLNGRV	99	DP-71	VGVVVTGRGAFDI	100
G93	DPL11	SSYTTRGTRV	101	DP7	GRGARDDGFDV	102
G95	L12a+	QQYSNYPLT	103	DP-75	GEIFDY	104
G101	DPL16	HSRDSSGNHVL	105	V2-1	GTSFSSSWYSNYFFYYYIDL	106
G102	DPL11	SSYTTRSTRV	107	DP-63	VGQYNYLHAFYLEY	108
G110	L12a+	QPYSNDALT	109	DP-75	GRSLTIG	110
G112	L12a+	QQSYSTPWT	111	DP-73	RWKGHFDY	112

Table 5.

Phage	Time	Output titre	Resistance Ω/cm^2	
			Before	After
Clone 65	30 min @ 4°C	$1x10^2$	613.3	
	30 min @ 37°C	$1.2x10^3$		493.3
Clone 73	30 min @ 4°C	$3.6x10^4$	640.0	
	30 min @ 37°C	$9.2x10^4$		486.7
Clone 77	30 min @ 4°C	$4.7x10^2$	593.3	
	30 min @ 37°C	$6.0x10^3$		506.7
Clone 93	30 min @ 4°C	$8.4x10^2$	586.7	
	30 min @ 37°C	$3.6x10^3$		470.0
pCANTAB6	30 min @ 4°C	$1.5x10^4$	626.7	
	30 min @ 37°C	$8.5x10^3$		526.7

Table 6.

Clone	RB	RB Asy	C	Tes	Ton	Kid	Ad C	Lu C	SM	Myo	Skin	Sp C
G65	+	-	-	-	-	-	-	-	-	-	-	-
G77	+	-	-	-	-	-	-	-	-	-	-	-
G102	+	-	-	-	-	-	-	-	-	-	-	-
G81	+	-	-	-	-	-	-	-	-	-	-	-
G101	+	-	-	-	-	-	-	-	-	-	-	-
G112	+	-	-	-	-	-	-	-	-	-	-	-
G85	+	-	-	-	-	-	-	-	-	-	-	-
G93	+	-	+	+	-	-	-	-	-	-	-	-
G79	+	-	-	-	-	-	-	-	-	-	-	-
G76	+	-	+	-	-	-	-	-	-	-	-	+
G67	+	-	+	-	+	+	-	-	-	-	-	-
G83	+	-	-	+	-	+	-	-	-	-	-	-
G92	+	-	-	+	-	-	-	-	+	-	-	+
G95	+	-	+	-	-	-	-	-	+	-	-	-
G73	+	-	+	-	-	-	-	-	-	-	-	+
G110	+	-	+	-	-	-	+	-	+	-	-	-
G88	+	-	-	-	-	-	-	+	-	-	-	-

Key: RB = rat brain; RB Asy = asymmetric staining of IL-1β-treated rat brain; C = cerebellum; Tes = testis; Ton = tonsil; Kid = kidney; Ad C = adenocarcinoma; Lu C = lung carcinoma; SM = striated muscle; Myo = myometrium; Lu = normal lung; Sp C = spinal cord; Lu = normal lung.

Table 7.

Phage	Titre added	Output after 30 min at 37 °C
D5	$1x10^8$	$6.2x10^4$
	$1x10^6$	$5.6x10^2$
	$1x10^4$	45
D5 + SAP	$1x10^8$	$1.1x10^5$
	$1x10^6$	$1.5x10^3$
	$1x10^4$	50
pCANTAB6	$1x10^8$	$2.6x10^3$
	$1x10^6$	20
	$1x10^4$	0
pCANTAB6 + SAP	$1x10^8$	$2.0x10^3$
	$1x10^6$	15
	$1x10^4$	0

CLAIMS

1. A mixture or panel of at least 5 different specific binding members each comprising an antibody VH variable domain and/or an antibody VL variable domain,

wherein an antibody VH variable domain has an amino acid sequence selected from the group consisting of antibody VH variable domains that comprise a VH CDR sequence shown in Table 4 (SEQ ID NO.'s 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110 and 112) and the G65 (SEQ ID NO. 2), G67 (SEQ ID NO. 6), G73 (SEQ ID NO. 10), G76 (SEQ ID NO. 14), G77 (SEQ ID NO. 18), G78 (SEQ ID NO. 22), G79 (SEQ ID NO. 24), G81 (SEQ ID NO. 30), G83 (SEQ ID NO. 34), G85 (SEQ ID NO. 38), G88 (SEQ ID NO. 42), G92 (SEQ ID NO. 46), G93 (SEQ ID NO. 50), G95 (SEQ ID NO. 54), G101 (SEQ ID NO. 58), G102 (SEQ ID NO. 62), G110 (SEQ ID NO. 66), G112 (SEQ ID NO. 70) and D5 (SEQ ID NO. 74) VH domain sequences disclosed herein, and/or

an antibody VL variable domain has an amino acid sequence selected from the group consisting of antibody VL variable domains that comprise a VL CDR sequence shown in Table 4 (SEQ ID NO.'s 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109 and 111) and the G65 (SEQ ID NO. 4), G67 (SEQ ID NO. 8), G73 (SEQ ID NO. 12), G76 (SEQ ID NO. 16), G77 (SEQ ID NO.20), G78 (SEQ ID NO. 24), G79 (SEQ ID NO. 28), G81 (SEQ ID NO. 32), G83 (SEQ ID NO. 36), G85 (SEQ ID NO. 40), G88 (SEQ ID NO. 44), G92 (SEQ ID NO. 48), G93 (SEQ ID NO. 52), G95 (SEQ ID NO. 56), G101 (SEQ ID NO. 60), G102 (SEQ ID NO. 64), G110 (SEQ ID NO. 68), G112 (SEQ ID NO. 72) and D5 (SEQ ID NO. 76) VL domain sequences disclosed herein,

each specific binding member being able, when displayed on the surface of filamentous bacteriophage particles or in the case of a specific binding member comprising the D5 VH

variable domain and/or D5 VL variable domain when bound to human serum amyloid protein, to pass through a mammalian blood brain barrier.

2. A mixture or panel according to claim 1 comprising at least 10 of said specific binding members.

3. A mixture or panel according to claim 1 wherein said blood brain barrier is a human or rat blood brain barrier.

4. A mixture or panel according to claim 3 which consists of 18 specific binding members, each specific binding members comprising a pair of respective antibody VH and VL variable domain sequences as follows:

	VH domain sequence	VL domain sequences
1)	G65 (SEQ ID NO. 2)	G65 (SEQ ID NO. 4);
2)	G67 (SEQ ID NO. 6)	G67 (SEQ ID NO. 8);
3)	G73 (SEQ ID NO. 10)	G73 (SEQ ID NO. 12);
4)	G76 (SEQ ID NO. 14)	G76 (SEQ ID NO. 16);
5)	G77 (SEQ ID NO. 18)	G77 (SEQ ID NO.20);
6)	G78 (SEQ ID NO. 22)	G78 (SEQ ID NO. 24);
7)	G79 (SEQ ID NO. 24)	G79 (SEQ ID NO. 28);
8)	G81 (SEQ ID NO. 30)	G81 (SEQ ID NO. 32);
9)	G83 (SEQ ID NO. 34)	G83 (SEQ ID NO. 36);
10)	G85 (SEQ ID NO. 38)	G85 (SEQ ID NO. 40);
11)	G88 (SEQ ID NO. 42)	G88 (SEQ ID NO. 44);
12)	G92 (SEQ ID NO. 46)	G92 (SEQ ID NO. 48);
13)	G93 (SEQ ID NO. 50)	G93 (SEQ ID NO. 52);
14)	G95 (SEQ ID NO. 54)	G95 (SEQ ID NO. 56);
15)	G101 (SEQ ID NO. 58)	G101 (SEQ ID NO. 60);

16) G102 (SEQ ID NO. 62) G102 (SEQ ID NO. 64);

17) G110 (SEQ ID NO. 66) G110 (SEQ ID NO. 68);

18) G112 (SEQ ID NO. 70) G112 (SEQ ID NO. 72).

5. A method of obtaining one or more specific binding members, the method comprising bringing into contact a mixture or panel of specific binding members according to any one of claims 1 to 4, and selecting one or more specific binding members of the mixture or panel able when displayed on filamentous bacteriophage particles to pass through a mammalian blood brain barrier.

6. A method according to claim 5 wherein specific binding members in the mixture or panel are displayed on the surface of filamentous bacteriophage particles, each filamentous bacteriophage particle containing nucleic acid encoding the specific binding member displayed on its surface.

7. A method according to claim 5 or claim 6 wherein selection on ability to pass through a blood brain barrier is followed by one or more further rounds of selection for ability to bind a type of cell of the brain or central nervous system (CNS)

8. A method according to claim 7 wherein said type of cell is selected from endothelial cells, cells of the meninges, parenchyma, choroid plexus, cerebrum, cerebellum, spinal cord and microglia.

9. A method according to claim 6 wherein nucleic acid is taken from a selected filamentous bacteriophage particle.

10. A method according to claim 9 wherein nucleic acid with a nucleotide sequence within the nucleic acid taken from a selected filamentous bacteriophage particle is used in subsequent production of a specific binding member or an antibody VH or VL variable domain.

11. A method according to any one of claims 5 to 10 comprising provision in isolated form of a selected specific binding member or antibody VH or VL domain of a selected specific binding member.

12. A method according to claim 11 wherein a selected specific binding member or an antibody VH or VL variable domain of a selected specific binding member in isolated form is formulated into a composition including at least one additional component.

13. A method according to any one of claims 5 to 12 wherein a selected specific binding member or antibody VH or VL domain of a selected specific binding member is provided in a fusion protein with additional amino acids.

14. A method according to claim 13 wherein said additional amino acids provide an antibody constant region.

15. A composition comprising a plurality of different specific binding members or antibody VH or VL variable domains obtainable from a mixture or panel according to any one of claims 1 to 4.

16. A specific binding member or antibody VH or VL variable domain obtainable from a mixture or panel according to any one of claims 1 to 3.

17. A specific binding member or antibody VH or VL variable domain obtainable from a mixture or panel according to claim 4.

18. A specific binding member according to claim 17 comprising the G93 antibody VH domain (SEQ ID NO. 50) and the G93 antibody VL domain (SEQ ID NO. 52).

19. A specific binding member according to claim 17 comprising the G73 antibody VH domain (SEQ ID NO. 10) and the G73 antibody VL domain (SEQ ID NO. 12).

20. A specific binding member according to claim 16 comprising the D5 antibody VH domain (SEQ ID NO. 74) and the D5 antibody VL domain (SEQ ID NO. 76).

21. A composition or specific binding member according to any one of claims 15 to 20 wherein a specific binding member or antibody VH or VL variable domain is in a fusion with additional amino acids.

22. A composition or specific binding member according to claim 21 wherein said additional amino acids provide an antibody constant region.

23. A composition according to or composition comprising a specific binding member or antibody VH or VL variable domain according to any one of claims 15 to 22, comprising a pharmaceutically acceptable excipient or carrier.

24. An isolated nucleic acid which comprises a nucleotide sequence encoding a specific binding member or antibody VH or VL domain according to any one of claims 16 to 22.

25. A host cell transformed with nucleic acid according to claim 24.

26. A method of producing a specific binding member or antibody VH or VL domain, the method comprising culturing host cells according to claim 25 under conditions for production of said specific binding member or antibody VH or VL domain.

27. A method according to claim 26 further comprising isolating and/or purifying said specific binding member or antibody VH or VL variable domain.

28. A method according to claim 26 or claim 27 further comprising formulating the specific binding member or antibody VH or VL variable domain into a composition including at least one additional component.

29. A method of obtaining a specific binding member with a desired property selected from ability to cross the blood brain barrier, specificity for a brain antigen and specificity for an endothelial cell antigen, the method comprising

providing by way of addition, deletion, substitution or insertion of one or more amino acids in the amino acid sequence of an antibody VH variable domain selected from the G65 (SEQ ID NO. 2), G67 (SEQ ID NO. 6), G73 (SEQ ID NO. 10), G76 (SEQ ID NO. 14), G77 (SEQ ID NO. 18), G78 (SEQ ID NO. 22), G79 (SEQ ID NO. 24), G81 (SEQ ID NO. 30), G83 (SEQ ID NO. 34), G85 (SEQ ID NO. 38), G88 (SEQ ID NO. 42), G92 (SEQ ID NO. 46), G93 (SEQ ID NO. 50), G95 (SEQ ID NO. 54), G101 (SEQ ID NO.

58), G102 (SEQ ID NO. 62), G110 (SEQ ID NO. 66), G112 (SEQ ID NO. 70) and D5 (SEQ ID NO. 74) VH domain sequences disclosed herein a VH domain which is an amino acid sequence variant of the VH domain, and combining one or more VH domain amino acid sequence variants thus provided with one or more VL domains to provide one or more VH/VL combinations; and/or

providing by way of addition, deletion, substitution or insertion of one or more amino acids in the amino acid sequence of an antibody VL variable domain selected from the G65 (SEQ ID NO. 4), G67 (SEQ ID NO. 8), G73 (SEQ ID NO. 12), G76 (SEQ ID NO. 16), G77 (SEQ ID NO.20), G78 (SEQ ID NO. 24), G79 (SEQ ID NO. 28), G81 (SEQ ID NO. 32), G83 (SEQ ID NO. 36), G85 (SEQ ID NO. 40), G88 (SEQ ID NO. 44), G92 (SEQ ID NO. 48), G93 (SEQ ID NO. 52), G95 (SEQ ID NO. 56), G101 (SEQ ID NO. 60), G102 (SEQ ID NO. 64), G110 (SEQ ID NO. 68), G112 (SEQ ID NO. 72) and D5 (SEQ ID NO. 76) VL domain sequences disclosed herein a VL domain which is an amino acid sequence variant of the VL domain, and combining one or more VL domain amino acid sequence variants thus provided with one or more VH domains to provide one or more VH/VL domain combinations;

and

testing the VH/VL combination or combinations for to identify a specific binding member with the desired property.

30. A method of obtaining a specific binding member with a desired property, which method comprises:

providing starting nucleic acids encoding one or more VH domains which either comprise a CDR3 to be replaced or lack a CDR3 encoding region, and combining said starting nucleic acid with a donor nucleic acid encoding an amino acid sequence substantially as set out in Table 4 for a VH CDR3 (SEQ ID NO.'s 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110 and 112) such that said donor nucleic acid

is inserted into the CDR3 region in the starting nucleic acid, so as to provide a product nucleic acids encoding VH domains; or

providing starting nucleic acids encoding one or more VL domains which either comprise a CDR3 to be replaced or lack a CDR3 encoding region, and combining said starting nucleic acid with a donor nucleic acid encoding an amino acid sequence substantially as set out in Table 4 for a VL CDR3 (SEQ ID NO.'s 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109 and 111) such that said donor nucleic acid is inserted into the CDR3 region in the starting nucleic acid, so as to provide a product nucleic acids encoding VL domains;

expressing the nucleic acids of said product nucleic acids encoding VH domains and combining the VH domains thus produced with one or more VL domains to provide VH/VL combinations, and/or expressing the nucleic acids of said product nucleic acids encoding VL domains and combining the VL domains thus produced with one or more VH domains to provide VH/VL combinations;

selecting a specific binding member comprising a VH/VL combination with the desired property; and

recovering said specific binding member with the desired property and/or nucleic acid encoding it.

31. A method according to claim 30 wherein the desired property is selected from ability to cross the blood brain barrier, ability bind an endothelial cell or other brain cell antigen, ability to bind areas of inflammation in the brain or blood brain barrier breakdown, ability to bind ICAM and ability to bind transferrin receptor.

32. A method according to any one of claims 29 to 31 further comprising formulating the specific binding member or an

antibody VH or VL variable domain thereof into a composition including at least one additional component.

33. A specific binding member according to any one of claims 16-20 or obtained using a method according to any one of claims 5-14, 26-32, which specific binding member is an scFv antibody molecule.

34. A specific binding member according to any one of claims 16-20 or obtained using a method according to any one of claims 5-14, 26-32, which specific binding member is a whole antibody.

35. A specific binding member according to claim 34 wherein said whole antibody is an IgG4 antibody.

36. A method comprising causing or allowing binding to an antigen a mixture or panel of specific binding members according to any one of claims 1 to 4, a composition or specific binding member according to any one of claims 15-23, 33-35, or a specific binding member obtained using a method according to any one of claims 5-14, 26-32.

37. A method according to claim 36 wherein said binding takes place *in vitro*.

38. A method according to claim 36 or claim 37 comprising determining the amount of binding of specific binding member to antigen.

SEQUENCE LISTING

G65 V_H

SEQ ID NO. 1 encoding SEQ ID NO. 2

```
CAGCTGGTGCAGTGTGGGGAGTCGTGGTACAGCCTGGGGGGGTCCCTGAGACTCTACTGT
Q  L  V  Q  C  G  E  S  W  Y  S  L  G  G  S  L  R  L  Y  C

GCATGCTCTGGATTCACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGC
A  C  S  G  F  T  F  S  S  Y  G  M  H  W  V  R  Q  A  P  G

AAGGGGCTGGAGTGGGTGGCAGTTATATCATATGATGGAAGTATTAAATACTATGCAGAC
K  G  L  E  W  V  A  V  I  S  Y  D  G  S  I  K  Y  Y  A  D

TCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAA
S  V  K  G  R  F  T  I  S  R  D  N  S  K  N  T  L  Y  L  Q

ATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGCGAACTGGTGAATAT
M  N  S  L  R  A  E  D  T  A  V  Y  Y  C  A  R  T  G  E  Y

AGTGGCTACGATACGAGTGGTGTGGAGCTCTGGGGCCAGGGAACCCTGGTCACCGTCTCC
S  G  Y  D  T  S  G  V  E  L  W  G  Q  G  T  L  V  T  V  S

TCA
S
```

G65 V_L

SEQ ID NO. 3 encoding SEQ ID NO. 4

```
TCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACA
S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T

TGCCAAGGAGACAGCCTCAGAAGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAG
C  Q  G  D  S  L  R  S  Y  Y  A  S  W  Y  Q  Q  K  P  G  Q

GCCCCTGTACTTGTCATCTGTGGTAATAACACCCGGCCCTCAGGGATCCCAGACCGATTC
A  P  V  L  V  I  C  G  N  N  T  R  P  S  G  I  P  D  R  F

TCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCCTCACTGGGGCTCAGGCGGAAGAT
S  G  S  S  S  G  N  T  A  S  L  T  L  T  G  A  Q  A  E  D

GAGGCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAACCATGTGGTATTCGGCGGA
E  A  D  Y  Y  C  N  S  R  D  S  S  G  N  H  V  V  F  G  G

GGGACCAAGCTGACCGTCCTAGGT
G  T  K  L  T  V  L  G
```

G67 V_H

SEQ ID NO. 5 encoding SEQ ID NO. 6

```
GAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTCAAGCCTGGAGGGTCCCTGAGACTC
E  V  Q  L  V  Q  S  G  G  G  L  V  K  P  G  G  S  L  R  L

TCCTGTGCAGCCTCTGGATTCACCTTCAGTGACTACTACATGCGTTGGGTCCGCCAGGCG
S  C  A  A  S  G  F  T  F  S  D  Y  Y  M  R  W  V  R  Q  A

CCAGGGAAGGGGCTGGAGTGGCTTTCATACATTAGTCCTGATTCTAGTATCACAAAATAC
P  G  K  G  L  E  W  L  S  Y  I  S  P  D  S  S  I  T  K  Y

GCAGACTCTGTGAAGGGCCGATTCACCATCTCCAGGGACAACGCCAAGAACACGCTGTAT
A  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  T  L  Y

CTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAAAGATTTC
L  Q  M  N  S  L  R  A  E  D  T  A  V  Y  Y  C  A  K  D  F

GTAGCTACTATGGTTCGGGGAGCCCCCACTAGGTACTTCGATCTCTGGGGCAGAGGGACA
V  A  T  M  V  R  G  A  P  T  R  Y  F  D  L  W  G  R  G  T

ATGGTCACCGTCTCGAGT
M  V  T  V  S  S
```

G67 V_L

SEQ ID NO. 7 encoding SEQ ID NO. 8

```
TCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACT
S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T

TGCCAAGGAGACAGTCTCAGAAGCTATTACACAAACTGGTTCCAGCAGAAGCCAGGACAG
C  Q  G  D  S  L  R  S  Y  Y  T  N  W  F  Q  Q  K  P  G  Q

GCCCCTCTACTTGTCGTCTATGCTAAAAATAAGCGGCCCTCAGGGATCCCAGACCGATTC
A  P  L  L  V  V  Y  A  K  N  K  R  P  S  G  I  P  D  R  F

TCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAAGAT
S  G  S  S  S  G  N  T  A  S  L  T  I  T  G  A  Q  A  E  D

GAGGCTGACTATTACTGTCATTCCCGGGACAGCAGTGGTAACCATGTGCTTTTCGGCGGA
E  A  D  Y  Y  C  H  S  R  D  S  S  G  N  H  V  L  F  G  G

GGGACCAAGCTGACCGTCCTAGGT
G  T  K  L  T  V  L  G
```

G73 V_H

SEQ ID NO. 9 encoding SEQ ID NO. 10

```
CAGGTGCAGCTGGTGCAATCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTC
Q  V  Q  L  V  Q  S  G  G  G  V  V  Q  P  G  R  S  L  R  L

TTCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCT
F  C  A  A  S  G  F  T  F  S  S  Y  G  M  H  W  V  R  Q  A

CCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATCATATGATGGAAGTATTAAATACTAT
P  G  K  G  L  E  W  V  A  V  I  S  Y  D  G  S  I  K  Y  Y

GCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
A  D  S  V  K  G  R  F  T  I  S  R  D  N  S  K  N  T  L  Y

CTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCGGTGTATTACTGTGCGCGAACTGGT
L  Q  M  N  S  L  R  A  E  D  T  A  V  Y  Y  C  A  R  T  G

GAATATAGTGGCTACGATACGAGTGGTGTGGAGCTCTGGGGCAGGAGGACAATGGTCACC
E  Y  S  G  Y  D  T  S  G  V  E  L  W  G  R  R  T  M  V  T

GTCTCTTCA
V  S  S
```

G73 V_L

SEQ ID NO. 11 encoding SEQ ID NO. 12

```
TCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACA
S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T

TGCCAAGGAGACAGCCTCAGAAGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAG
C  Q  G  D  S  L  R  S  Y  Y  A  S  W  Y  Q  Q  K  P  G  Q

GCCCCTGTACTTGTCATCTATGGTAATAACAACCGGCCCTCAGGGATCCCAGACCGATTC
A  P  V  L  V  I  Y  G  N  N  N  R  P  S  G  I  P  D  R  F

TCTGGCTCCAGCTCAGGATACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAAGAT
S  G  S  S  S  G  Y  T  A  S  L  T  I  T  G  A  Q  A  E  D

GAGGCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAACCATGTGGTATTCGGCGGA
E  A  D  Y  Y  C  N  S  R  D  S  S  G  N  H  V  V  F  G  G

GGGACCAAGCTGACCGTCCTAGGT
G  T  K  L  T  V  L  G
```

G76 V_H

SEQ ID NO. 13 encoding SEQ ID NO. 14

```
CAGGTGCAGCTACAGCAGTGGGGGCCAGGACAGGTGAAGCCTTGGGAGACCCTGTCACTC
Q  V  Q  L  Q  Q  W  G  P  G  Q  V  K  P  W  E  T  L  S  L

ACCTGCAGTATCTCTGGTGGCTACATCAATTCGCACTACTGGAATTGGATCAGACAGCCC
T  C  S  I  S  G  G  Y  I  N  S  H  Y  W  N  W  I  R  Q  P

CCAGGCAAGGGACTCGAGTGGATTGGATATATCTATTACAGTGGGACCACCAACTACAAC
P  G  K  G  L  E  W  I  G  Y  I  Y  Y  S  G  T  T  N  Y  N

CCCTCCCTCAAGAGTCGAGTCTCTATATCAATAGACACGTCCAAGAACCAGGTCTCCCTA
P  S  L  K  S  R  V  S  I  S  I  D  T  S  K  N  Q  V  S  L

AAGTTGAGTTCTGTGATCGCCGCAGACACGGCCGTCTATTTTTGTGCGAGAGGTCCATTA
K  L  S  S  V  I  A  A  D  T  A  V  Y  F  C  A  R  G  P  L

CGATTACGCGGCTTTTGATCTGTGGGGCCAAGGCACCCTGGTCACCGTCTCGAGT
R  L  R  A  F  D  L  W  G  Q  G  T  L  V  T  V  S  S
```

G76 V_L

SEQ ID NO. 15 encoding SEQ ID NO. 16

```
TCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACA
S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T

TGCCAAGGAGACAGCCTCAGAAGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAG
C  Q  G  D  S  L  R  S  Y  Y  A  S  W  Y  Q  Q  K  P  G  Q

GCCCCTGTACTTGTCATCTATGGTAAAAACAACCGGCCCTCAGGGATCCCAGACCGATTC
A  P  V  L  V  I  Y  G  K  N  N  R  P  S  G  I  P  D  R  F

TCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAAGAT
S  G  S  S  S  S  G  N  T  A  S  L  T  I  T  G  A  Q  A  E  D

GAGGCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAACCATGTGGTATTCGGCGGA
E  A  D  Y  Y  C  N  S  R  D  S  S  G  N  H  V  V  F  G  G

GGGACCAAGCTGACCGTCCTAGGT
G  T  K  L  T  V  L  G
```

G77 V_H

SEQ ID NO. 17 encoding SEQ ID NO. 18

```
GAGGTCCAGCTGGGACAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTC
E  V  Q  L  G  Q  S  G  A  E  V  K  K  P  G  A  S  V  K  V

TCCTGTAAGGTTTCCGGAGACACCCTCAGTGAATTCTCCATACACTGGGTGCGACAGGCT
S  C  K  V  S  G  D  T  L  S  E  F  S  I  H  W  V  R  Q  A

CCTGGAAAAGGCCTTGAGTGGATGGGAGGTTTCGATCCTGAAGATGTTGAGATTACCTAC
P  G  K  G  L  E  W  M  G  G  F  D  P  E  D  V  E  I  T  Y

GCACAGAAGTTTCAGGGCAGAGTCACCATGACCGAGGACACATCTACAGAGACAGCCTAC
A  Q  K  F  Q  G  R  V  T  M  T  E  D  T  S  T  E  T  A  Y

ATGGAGCTGCGCAGCCTGAGACCTGAGGACACGGCCGTGTATTATTGTGCAACTGCCTCT
M  E  L  R  S  L  R  P  E  D  T  A  V  Y  Y  C  A  T  A  S

GAGACCCGGGTCATGGTTGATGATGTTTTTAATGTCTGGGGCCGAGGGACAATGGTCACC
E  T  R  V  M  V  D  D  V  F  N  V  W  G  R  G  T  M  V  T

GTCTCGAGT
V  S  S
```

G77 V_L

SEQ ID NO. 19 encoding SEQ ID NO. 20

```
CAGTCTGTGTTGACGCAGCCGCCCTCAGTGTCTGGGACCCCCGGGCAGAGAGTCACCATC
Q  S  V  L  T  Q  P  P  S  V  S  G  T  P  G  Q  R  V  T  I

TCTTGTTCTGGAGGCAGATCCAACATCGGCAGTAATACTGTAAAGTGGTATCAGCAGCTC
S  C  S  G  G  R  S  N  I  G  S  N  T  V  K  W  Y  Q  Q  L

CCAGGAACAGCCCCCAAACTCCTCATCTATGGTAACAGCAATCGGCCCTCAGGGGTCCCT
P  G  T  A  P  K  L  L  I  Y  G  N  S  N  R  P  S  G  V  P

GACCGCTTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCAGTGGGCTCCAG
D  R  F  S  G  S  K  S  G  T  S  A  S  L  A  I  S  G  L  Q

GCTGAGGATGAGGCTCATTATTACTGCCAGTCCTATGACAGCAGCCTGAGTAATATGATA
A  E  D  E  A  H  Y  Y  C  Q  S  Y  D  S  S  L  S  N  M  I

TTCGGCGAAGGGACCAAGCTGACCGTCCTAGGT
F  G  E  G  T  K  L  T  V  L  G
```

G78 V_H

SEQ ID NO. 21 encoding SEQ ID NO. 22

```
CAGGGGCAGCTACAGGAGTGGGGGCCAAGACAGGTGAAGCCTTGGGAGACCCTGGCACTC
Q  G  Q  L  Q  E  W  G  P  R  Q  V  K  P  W  E  T  L  A  L

ACCTGCAGTATCTTTGGTGGCTACATCAATTCGTACTACTGGAATTGGATCACACAGCCC
T  C  S  I  F  G  G  Y  I  N  S  Y  Y  W  N  W  I  T  Q  P

TCAGGCAAGGGACTCGAGTGGATTGGATATATCTATTACAGTGGGACCACCAACTACAAC
S  G  K  G  L  E  W  I  G  Y  I  Y  Y  S  G  T  T  N  Y  N

CCCTCCCTCAAGAGTCGAGTCTCTATATCAATAGACACGTACAAGAACCAGGTCTCCCTA
P  S  L  K  S  R  V  S  I  S  I  D  T  Y  K  N  Q  V  S  L

AAGTTGAGTTCTGAGATCTGCCGCAGTACACGTGCCGTCTATTTTTGTGCGAGAGGTCCA
K  L  S  S  E  I  C  R  S  T  R  A  V  Y  F  C  A  R  G  P

TTACGATTACGCGCTTTTGATCTGTGGGGCCAAGGCACCCTGGTCTCCGTCTCGAGT
L  R  L  R  A  F  D  L  W  G  Q  G  T  L  V  S  V  S  S
```

G78 V_L

SEQ ID NO. 23 encoding SEQ ID NO. 24

```
GAGCTGACTCAGGACCTTGCTGTGTCTGTGGCCTTGGCACTGACAGTCAGGATCACATGC
E  L  T  Q  D  L  A  V  S  V  A  L  A  L  T  V  R  I  T  C

CAAGTAGACAGCCTCAATAGCTATTATGCCAGCTGGTGCCAGCAGAAGCCAGCACAGGCC
Q  V  D  S  L  N  S  Y  Y  A  S  W  C  Q  Q  K  P  A  Q  A

CCTGCACTTGTCATCTATGGTAAAAACTCCCGGCCCTCAGGGATCCCAGCCCGATTCTCT
P  A  L  V  I  Y  G  K  N  S  R  P  S  G  I  P  A  R  F  S

GGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTAGGGCTCAGGCGGAAGATGAG
G  S  S  S  G  N  T  A  S  L  T  I  T  R  A  Q  A  E  D  E

GCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAACCATGTGGTATTCGGCGGAGGG
A  D  Y  Y  C  N  S  R  D  S  S  G  N  H  V  V  F  G  G  G

ACCAAGCTGACCGTCCTAGGT
T  K  L  T  V  L  G
```

G79 V_H

SEQ ID NO. 25 encoding SEQ ID NO. 26

```
CAGGTGCAGCTGGTGCAGTCTGCGGGTGACCTGCAGAAGCCTGGGGCCTCAGTGAAGGTT
Q  V  Q  L  V  Q  S  A  G  D  L  Q  K  P  G  A  S  V  K  V

TCCTGCAAGACATTTGGATACAGCTTCAGCAGTTACCATATACACTGGGTGAGACAGGCC
S  C  K  T  F  G  Y  S  F  S  S  Y  H  I  H  W  V  R  Q  A

CCTGGACAAGGGCTTGAGTGGATGGGGATAATCGACCCTCGTGGTGGCAGTACAAGTTAC
P  G  Q  G  L  E  W  M  G  I  I  D  P  R  G  G  S  T  S  Y

GCACAGAAGTTCCAGGGCAGAGTCACCATGACCGCTGACACGTCCACAGGCACAGTGTAT
A  Q  K  F  Q  G  R  V  T  M  T  A  D  T  S  T  G  T  V  Y

ATGGAACTGAGCAGCCTGAAATCTGACGACACGTCCATATATTACTGTGCAAGGAGGTCC
M  E  L  S  S  L  K  S  D  D  T  S  I  Y  Y  C  A  R  R  S

GGGGCTCTTGGGGGACTAATTCCCCTTAACTACTTTGACTACTGGGGCCGGGGGACAATG
G  A  L  G  G  L  I  P  L  N  Y  F  D  Y  W  G  R  G  T  M

GTCACCGTCTCGAGT
V  T  V  S  S
```

G79 V_L

SEQ ID NO. 27 encoding SEQ ID NO. 28

```
TCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACT
S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T

TGCCAAGGAGACAGTCTCAGAAGCTATTACACAAACTGGTTCCAGCAGAAGCCAGGACAG
C  Q  G  D  S  L  R  S  Y  Y  T  N  W  F  Q  Q  K  P  G  Q

GCCCCTCTACTTGTCGTCTATGCTAAAAATAAGCGGCCCTCAGGGATCCCAGACCGATTC
A  P  L  L  V  V  Y  A  K  N  K  R  P  S  G  I  P  D  R  F

TCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAAGAT
S  G  S  S  S  G  N  T  A  S  L  T  I  T  G  A  Q  A  E  D

GAGGCTGACTATTACTGTCATTCCCGGGACAGCAGTGGTAACCATGTGCTTTTCGGCGGA
E  A  D  Y  Y  C  H  S  R  D  S  S  G  N  H  V  L  F  G  G

GGGACCAAGCTGACCGTCCTAGGT
G  T  K  L  T  V  L  G
```

G81 V_H

SEQ ID NO. 29 encoding SEQ ID NO. 30

```
CAGGTCACCTTGAAGGAGTCTGGGCCTACGCTGGTGAAACCCACACAGACCCTTACGCTG
Q  V  T  L  K  E  S  G  P  T  L  V  K  P  T  Q  T  L  T  L

ACCTGCACCTTCTCTGGGTTCTCACTCAGGACTACTGGAGTGGGCGTGGGCTGGGTCCGT
T  C  T  F  S  G  F  S  L  R  T  T  G  V  G  V  G  W  V  R

CAGCCCCCAGGAGAGGCCCTGGAGTCGCTTGCGATCATTTATTGGAATGATGATAAGCGC
Q  P  P  G  E  A  L  E  S  L  A  I  I  Y  W  N  D  D  K  R

AACAGCCCATCTCTGAAGAGAAGGCTCACCATCACCAAGGACACCTCCAGAAACCAGGTG
N  S  P  S  L  K  R  R  L  T  I  T  K  D  T  S  R  N  Q  V

GTCCTTACAATGACCAACATGGACCCTGTGGACACAGCCACATATTACTGTGCCCGCGTG
V  L  T  M  T  N  M  D  P  V  D  T  A  T  Y  Y  C  A  R  V

ACCAATGGTCACTGGTACTACTTTGACTACTGGGGCAGAGGCACCCTGGTCACCGTCTCG
T  N  G  H  W  Y  Y  F  D  Y  W  G  R  G  T  L  V  T  V  S

AGT
S
```

G81 V_L

SEQ ID NO. 31 encoding SEQ ID NO. 32

```
CAGTCTGTGCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATC
Q  S  V  L  T  Q  P  A  S  V  S  G  S  P  G  Q  S  I  T  I

TCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTATGTCTCCTGGTACCAACAA
S  C  T  G  T  S  S  D  V  G  G  Y  N  Y  V  S  W  Y  Q  Q

CACCCAGGCAAAGCCCCCAAACTCATGATTTATGAGGGCAGTAAGCGGCCCTCAGGGGTT
H  P  G  K  A  P  K  L  M  I  Y  E  G  S  K  R  P  S  G  V

TCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCTTGACCATCACTGGGGCT
S  N  R  F  S  G  S  K  S  G  N  T  A  S  L  T  I  T  G  A

CAGGCGGAAGATGAGGCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAACCATGTG
Q  A  E  D  E  A  D  Y  Y  C  N  S  R  D  S  S  G  N  H  V

GTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT
V  F  G  G  G  T  K  L  T  V  L  G
```

G83 V_H

SEQ ID NO. 33 encoding SEQ ID NO. 34

```
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
Q  V  Q  L  Q  E  S  G  P  G  L  V  K  P  S  E  T  L  S  L

ACCTGCACTGTCTCTGGTGGTTCCATCAGTAGTTACTACTGGAGCTGGATCCGGCAGCCC
T  C  T  V  S  G  G  S  I  S  S  Y  Y  W  S  W  I  R  Q  P

CCAGGGAAGGGACTGGAGTGGATTGGGCATATCTATTACAGTGGGAACACCAACTACAAC
P  G  K  G  L  E  W  I  G  H  I  Y  Y  S  G  N  T  N  Y  N

CCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCGAAGAACCAGTTCTCCCTG
P  S  L  K  S  R  V  T  I  S  V  D  T  S  K  N  Q  F  S  L

AAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGGGCGGCGAGT
K  L  S  S  V  T  A  A  D  T  A  V  Y  Y  C  A  R  A  A  S

CTATCTTGTACTGGTAGCAGCTGCAGGTACAACTACTTCGACCCCTGGGGCCGAGGAACC
L  S  C  T  G  S  S  C  R  Y  N  Y  F  D  P  W  G  R  G  T

CTGGTCACCGTCTCGAGT
L  V  T  V  S  S
```

G83 V_L

SEQ ID NO. 35 encoding SEQ ID NO. 36

```
GACATCGTGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACAGAGTCACC
D  I  V  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R  V  T

ATCACTTGCCGGGCCAGTCAGGGTATTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCA
I  T  C  R  A  S  Q  G  I  S  S  W  L  A  W  Y  Q  Q  K  P

GGGAGAGCCCTTAAGGTCTTGATCTATAAGGCATGGCCTTTAGAAAGTGGGGTCCCATCA
G  R  A  L  K  V  L  I  Y  K  A  W  P  L  E  S  G  V  P  S

AGGTTCACCGACAGTGGATCTGGGGCAGATTTCACTCTCACCATCAGCAGTTTGCAACCT
R  F  T  D  S  G  S  G  A  D  F  T  L  T  I  S  S  L  Q  P

GAAGATTTTGCAACTTATTACTGTCAGCAGAGTTACAGTACTCCGTGGACGTTCGGCCAA
E  D  F  A  T  Y  Y  C  Q  Q  S  Y  S  T  P  W  T  F  G  Q

GGGACCAAGCTGAAGATCAAACGCCGG
G  T  K  L  K  I  K  R  R
```

G85 V_H

SEQ ID NO. 37 encoding SEQ ID NO. 38

```
CAGCTGCAGCTGCAGGAGTCCGGGGCTGAGCTTGTGAAGCCTGGGGCTTCAGTGAAAATG
Q  L  Q  L  Q  E  S  G  A  E  L  V  K  P  G  A  S  V  K  M

TCCTGCAAGGCTTCTGGCTATACCTTCGCCAGCTACTGGATAAACTGGATGTAGCAGAGG
S  C  K  A  S  G  Y  T  F  A  S  Y  W  I  N  W  M  Q  Q  R

CCTGGACAAGGCCTTGAGTGGATTGGACATATTTATCCTGTTAGAAGTATTACTAAGTAC
P  G  Q  G  L  E  W  I  G  H  I  Y  P  V  R  S  I  T  K  Y

AATGAGAAGTTCAAGAGTAAGGCCACACTGTCTCTAGACACATCCTCCAGCACAGCCTAC
N  E  K  F  K  S  K  A  T  L  S  L  D  T  S  S  S  T  A  Y

ATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTTCAAGAGGGGAC
M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  S  R  G  D

GGCAGTGATTATTATGCTATGGACTACTGGGGCCAGGGGACAATGGTCACCGTCTCTTCA
G  S  D  Y  Y  A  M  D  Y  W  G  Q  G  T  M  V  T  V  S  S
```

G85 V_L

SEQ ID NO. 39 encoding SEQ ID NO. 40

```
GATGTTGTGATGACTCAGTCTCCATCTTCCGTGTCTGCATCTGTAGGAGACAGAGTCACC
D  V  V  M  T  Q  S  P  S  S  V  S  A  S  V  G  D  R  V  T

ATCACTTGTCGGGCGAGTCAGGGTATTAGCAGTTATTTAGCCTGGTATCAGCAAAAACCA
I  T  C  R  A  S  Q  G  I  S  S  Y  L  A  W  Y  Q  Q  K  P

GGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCACTTTGCAATCAGGGGTCCCATCT
G  K  A  P  K  L  L  I  Y  A  A  S  T  L  Q  S  G  V  P  S

CGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCACCCTGCAGCCT
R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  T  L  Q  P

GAAGATGTTGCAACTTATTACTGTGAAAACTATAACAGTGTCCCGCTCAGTTTCGGCGGA
E  D  V  A  T  Y  Y  C  E  N  Y  N  S  V  P  L  S  F  G  G

GGGACCAAGCTGGAGATCAGACGT
G  T  K  L  E  I  R  R
```

G88 V_H

SEQ ID NO. 41 encoding SEQ ID NO. 42

```
GAGGTCCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTC
E  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  S  S  V  K  V

TCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCGACAGGCC
S  C  K  A  S  G  G  T  F  S  S  Y  A  I  S  W  V  R  Q  A

CCTGGACAAGGGCTTGAGTGGATGGGAAGGGATCATCCCTATCTTTGGACAGCAAACTAC
P  G  Q  G  L  E  W  M  G  R  D  H  P  Y  L  W  T  A  N  Y

GCACAGAAGTTCCAGGCAGAGTCACGATTACCGCGGGACGAATCCACGAGCACAGCCTAC
A  Q  K  F  Q  A  E  S  R  L  P  R  D  E  S  T  S  T  A  Y

ATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGAGAAGGG
M  E  L  S  S  L  R  S  E  D  T  A  V  Y  Y  C  A  R  E  G

ACTGCTATCCACCGCGCTTTTGATATCTGGGGGCAGGGGACCACGGTCACCGTCTCGAGC
T  A  I  H  R  A  F  D  I  W  G  Q  G  T  T  V  T  V  S  S
```

G88 V_L

SEQ ID NO. 43 encoding SEQ ID NO. 44

```
GACATCTAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTATTGGAGACAGAGTCACC
D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  I  G  D  R  V  T

ATCACCTGCCGGGCCAGTGAGGGTATTTATCACTGGTTGGCCTGGTATCAGCAGAAGCCA
I  T  C  R  A  S  E  G  I  Y  H  W  L  A  W  Y  Q  Q  K  P

GGGAAAGCCCCTAAACTCCTGATCTATAAGGCCTCTAGTTTAGCCAGTGGGGCCCCATCA
G  K  A  P  K  L  L  I  Y  K  A  S  S  L  A  S  G  A  P  S

AGGTTCAGCGGCAGTGGATTTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCT
R  F  S  G  S  G  F  G  T  D  F  T  L  T  I  S  S  L  Q  P

GATGATTTTGCAACTTATTACTGCCAACAATATAGTAATTATCCGCTCACTTTCGGCGGA
D  D  F  A  T  Y  Y  C  Q  Q  Y  S  N  Y  P  L  T  F  G  G

GGGACCAAGCTGGAGATCAAACGT
G  T  K  L  E  I  K  R
```

G92 V_H

SEQ ID NO. 45 encoding SEQ ID NO. 46

```
GAGGTGCAGCTGGTGGAGTCTGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
E  V  Q  L  V  E  S  G  P  G  L  V  K  P  S  E  T  L  S  L

ACCTGCACTGTCTCTGGTGGTTCCATCAGTAGTTACTACTGGAGCTGGATCCGGCAGCCC
T  C  T  V  S  G  G  S  I  S  S  Y  Y  W  S  W  I  R  Q  P

CCAGGGAAGGGACTGGAGTGGATTGGCTATATCTATTACAGTGGGAGCACCAACTACAAC
P  G  K  G  L  E  W  I  G  Y  I  Y  Y  S  G  S  T  N  Y  N

CCCCCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCATTTCTCCCTG
P  P  L  K  S  R  V  T  I  S  V  D  T  S  K  N  H  F  S  L

AAGCTGAACTCTGTGACCGCCGCAGACACGGCCGTGTATTACTGTGCGAGAGTTGGGGTG
K  L  N  S  V  T  A  A  D  T  A  V  Y  Y  C  A  R  V  G  V

GTGGTGACTGGGAGAGGTGCTTTTGATATCTGGGGGCCAAGGACAATGGTCACCGTCTCG
V  V  T  G  R  G  A  F  D  I  W  G  P  R  T  M  V  T  V  S

AGT
S
```

G92 V_L

SEQ ID NO. 47 encoding SEQ ID NO. 48

```
CAGTCTGTGCTGACTCAGCCACCCTCAGCGTCTGGGACCCCCGGGCAGAGGGTCACCATC
Q  S  V  L  T  Q  P  P  S  A  S  G  T  P  G  Q  R  V  T  I

TCTTGTTCTGGAAGCAGCTCCAACATCGGGAGTAACACTGTAAACTGGTACCAGCGACTC
S  C  S  G  S  S  S  N  I  G  S  N  T  V  N  W  Y  Q  R  L

CCAGGAGCGGCCCCCCAACTCCTCATCTACAATAATGACCAGCGGCCCTCAGGGATCCCT
P  G  A  A  P  Q  L  L  I  Y  N  N  D  Q  R  P  S  G  I  P

GACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGGCTCCCTGGTCATCAGTGGGCTCCAG
D  R  F  S  G  S  K  S  G  T  S  G  S  L  V  I  S  G  L  Q

TCTGAAGATGAGGCTGATTACTACTGTGCGTCATGGGATGACAGTCTGAATGGCCGGGTG
S  E  D  E  A  D  Y  Y  C  A  S  W  D  D  S  L  N  G  R  V

TTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT
F  G  G  G  T  K  L  T  V  L  G
```

G93 V_H

SEQ ID NO. 49 encoding SEQ ID NO. 50

```
CAGGTGCAGTTACAGCAGTCGGGGGCTGCGGTGAAGACGCCTGGGGCCTCAGTCAAAGTT
Q  V  Q  L  Q  Q  S  G  A  A  V  K  T  P  G  A  S  V  K  V

TCCTGTAAGGCATCTGGATATCCCTTCATCACCTACAACATGCACTGGGTGCGGCAGGCC
S  C  K  A  S  G  Y  P  F  I  T  Y  N  M  H  W  V  R  Q  A

CCTGGACAGGGCTTTGAGTGGATGGGAATAATCGACCCAAGTGGTGGTCGCACAACGTAC
P  G  Q  G  F  E  W  M  G  I  I  D  P  S  G  G  R  T  T  Y

TCAAAGAACTTCCAGGGCAGACTCACCGTGACCAGGGAGACGTCAACGACCACGGTCACC
S  K  N  F  Q  G  R  L  T  V  T  R  E  T  S  T  T  T  V  T

ATGGAGCTGAGTGGCCTGAGATCTGAGGACACGGCCCTGTATTTCTGTGCGAGAGGGCGC
M  E  L  S  G  L  R  S  E  D  T  A  L  Y  F  C  A  R  G  R

GGTGCGAGGGATGATGGTTTTGATGTCTGGGGCCAGGGGACAATGGTCACCGTCTCTTCA
G  A  R  D  D  G  F  D  V  W  G  Q  G  T  M  V  T  V  S  S
```

G93 V_L

SEQ ID NO. 51 encoding SEQ ID NO. 52

```
CAGTCTGTGCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATC
Q  S  V  L  T  Q  P  A  S  V  S  G  S  P  G  Q  S  I  T  I

TCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTATGTCTCCTGGTACCAACAA
S  C  T  G  T  S  S  D  V  G  G  Y  N  Y  V  S  W  Y  Q  Q

CACCCAGGCAAAGCCCCCAAACTCATGATTTATGAGGGCAGTAAGCGGCCCTCAGGGGTT
H  P  G  K  A  P  K  L  M  I  Y  E  G  S  K  R  P  S  G  V

TCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACAATCTCTGGGCTC
S  N  R  F  S  G  S  K  S  G  N  T  A  S  L  T  I  S  G  L

CAGGCTGAGGACGAGGCTGATTATTACTGCAGCTCATATACAACCAGGGGCACTCGAGTT
Q  A  E  D  E  A  D  Y  Y  C  S  S  Y  T  T  R  G  T  R  V

TTCGGCGGAGGGACCAAGCTGACCGTCCTAGGG
F  G  G  G  T  K  L  T  V  L  G
```

G95 V_H

SEQ ID NO. 53 encoding SEQ ID NO. 54

```
CAGGTACAGCTGCAGCAGTCAGGACCTGAGCTGGCGAGTCCTGGGGCATCAGTGACACTG
Q  V  Q  L  Q  Q  S  G  P  E  L  A  S  P  G  A  S  V  T  L

TCCTGCAAGGCTTCTGGCTACACATTTACTGACCATATTATGAATTGGGTAAAAAAGAGG
S  C  K  A  S  G  Y  T  F  T  D  H  I  M  N  W  V  K  K  R

CCTGGACAGGGCCTTGAGTGGATTGGAAGGATTTATCCAGTAAGTGGTGAAAGTAACTAC
P  G  Q  G  L  E  W  I  G  R  I  Y  P  V  S  G  E  S  N  Y

AATCAAAAGTTCATGGGCAAGGCCACATTCTCTGTAGACCGGTCCTCCAGCACGGTGTAT
N  Q  K  F  M  G  K  A  T  F  S  V  D  R  S  S  S  T  V  Y

ATGGTGTTGAACAGCCTGACATCTGAAGACCCTGCTGTCTATTACTGTGCAAGGGGGGAG
M  V  L  N  S  L  T  S  E  D  P  A  V  Y  Y  C  A  R  G  E

ATCTTTGACTATTGGGCCGGGGGACCACGGTCACCGTCTCCTTCA
I  F  D  Y  W  A  G  G  P  R  S  P  S  P  S
```

G95 V_L

SEQ ID NO. 55 encoding SEQ ID NO. 56

```
GACATCCAGATGACCCAGTCTCCTTCCACCCCGTCTGCATCTATTGGAGACAGAGTCACC
D  I  Q  M  T  Q  S  P  S  T  P  S  A  S  I  G  D  R  V  T

ATCACCTGCCGGGCCAGTGAGGGTATTTATCACTGGTTGGCCTGGTATCAGCAGAAGCCA
I  T  C  R  A  S  E  G  I  Y  H  W  L  A  W  Y  Q  Q  K  P

GGGAAAGCCCCTAAACTCCTGATCTATAAGGCCTCTAGTTTAGCCAGTGGGGCCCCATCA
G  K  A  P  K  L  L  I  Y  K  A  S  S  L  A  S  G  G  A  P  S

AGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCT
R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  Q  P

GATGATTTTGCAACTTATTACTGCCAACAATATAGTAATTATCCGCTCACTTTCGGCGGA
D  D  F  A  T  Y  Y  C  Q  Q  Y  S  N  Y  P  L  T  F  G  G

GGGACACGACTGGAGATTAAACGT
G  T  R  L  E  I  K  R
```

G101 V_H

SEQ ID NO. 57 encoding SEQ ID NO. 58

```
CAGGTGCAGCTGCAGGAGTCGGGCCCGGGACTGCTGAAGGCTTCGGAGACCCTGTCCCTC
Q  V  Q  L  Q  E  S  G  P  G  L  L  K  A  S  E  T  L  S  L

GTTTGCAGTGTCTCTGGCGCCTCCGTCGACAGTGACCAGTTCTACTGGGTCTGGATCCGT
V  C  S  V  S  G  A  S  V  D  S  D  Q  F  Y  W  V  W  I  R

CAGCCCCCAGGGAAAGGACTAGAGTGGATTGGGACTGCCTATTATAGTGGGAGCAGCCAC
Q  P  P  G  K  G  L  E  W  I  G  T  A  Y  Y  S  G  S  S  H

TACAACCCGTCCCTCAACAATCGGGTCACCATATCTGTAGACACGTCCAAAGACCTCTTC
Y  N  P  S  L  N  N  R  V  T  I  S  V  D  T  S  K  D  L  F

TCCCTGAGTCTGAACTCTGTGACCGTCGCAGATACGGCTGTGTATTACTGTGCGAGGGGG
S  L  S  L  N  S  V  T  V  A  D  T  A  V  Y  Y  C  A  R  G

ACTTCATTTAGCAGCAGTTGGTACAGTAATTACTTCTTCTATTATTACATTGACCTCTGG
T  S  F  S  S  S  W  Y  S  N  Y  F  F  Y  Y  Y  I  D  L  W

GGCAAGGGAACCCTGGTCACCGTCTCGAGT
G  K  G  T  L  V  T  V  S  S
```

G101 V_L

SEQ ID NO. 59 encoding SEQ ID NO. 60

```
TCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACT
S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T

TGCCAAGGAGACAGTCTCAGAAGCTATTACACAAACTGGTTCCAGCAGAAGCCAGGACAG
C  Q  G  D  S  L  R  S  Y  Y  T  N  W  F  Q  Q  K  P  G  Q

GCCCCTCTACTTGTCGTCTATGCTAAAAATAAGCGGCCCTCAGGGATCCCAGACCGATTC
A  P  L  L  V  V  Y  A  K  N  K  R  P  S  G  I  P  D  R  F

TCTGGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAAGAT
S  G  S  S  S  G  N  T  A  S  L  T  I  T  G  A  Q  A  E  D

GAGGCTGACTATTACTGTCATTCCCGGGACAGCAGTGGTAACCATGTGCTTTTCGGCGGA
E  A  D  Y  Y  C  H  S  R  D  S  S  G  N  H  V  L  F  G  G

GGGACCAAGCTGACCGTCCTAGGA
G  T  K  L  T  V  L  G
```

G102 V_H

SEQ ID NO. 61 encoding SEQ ID NO. 62

```
CAGGTGCAGCTACAGCAGTGGGGCGCGGGAGTACTGGAGCCTTCGGAGACCCTGTCCCTC
Q  V  Q  L  Q  Q  W  G  A  G  V  L  E  P  S  E  T  L  S  L

ACCTGCGGTGTTTCTGGTGCGTCTTTGGATGGTCACTACTGGGCCTGGATCCGCCAGTCC
T  C  G  V  S  G  A  S  L  D  G  H  Y  W  A  W  I  R  Q  S

CCAGGGACGGGGCTGGAATGGATTGCTGAAATCAATTCAAGTGGCAGCACCAACTACAAC
P  G  T  G  L  E  W  I  A  E  I  N  S  S  G  S  T  N  Y  N

CCGTCCCTCAAGAGCCGGGTCGCCATATCAATGGACACGTCTAAGAACTCCTTGTCCCTG
P  S  L  K  S  R  V  A  I  S  M  D  T  S  K  N  S  L  S  L

AACTTGAAGTCTGTGACCGCCGCGGACACGGCTGTATATTACTGTGCGAGAGTCGGGCAA
N  L  K  S  V  T  A  A  D  T  A  V  Y  Y  C  A  R  V  G  Q

TACAATTATCTCCACGCGTTCTACTTAGAATACTGGGGCCGAAGGACAATGGTCACCGTC
Y  N  Y  L  H  A  F  Y  L  E  Y  W  G  R  R  T  M  V  T  V

TCTTCA
S  S
```

G102 V_L

SEQ ID NO. 63 encoding SEQ ID NO. 64

```
CAGTCTGTGCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGGCAGTCGATCACCATC
Q  S  V  L  T  Q  P  A  S  V  S  G  S  P  G  Q  S  I  T  I

TCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTATGTCTCCTGGTACCAACAA
S  C  T  G  T  S  S  D  V  G  G  Y  N  Y  V  S  W  Y  Q  Q

CACCCAGGCAAAGCCCCCAAACTCATGATTTATGAGGGCAGTAAGCGGCCCTCAGGGGTT
H  P  G  K  A  P  K  L  M  I  Y  E  G  S  K  R  P  S  G  V

TCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACAATCTCTGGGCTC
S  N  R  F  S  G  S  K  S  G  N  T  A  S  L  T  I  S  G  L

CAGGCTGAGGACGAGGCTGATTATTACTGCAGCTCATATACAACCAGGAGCACTCGAGTT
Q  A  E  D  E  A  D  Y  Y  C  S  S  Y  T  T  R  S  T  R  V

TTCGGCGGAGGGACCAAGCTGACCGTCCTAGGA
F  G  G  G  T  K  L  T  V  L  G
```

G110V_H

SEQ ID NO. 65 encoding SEQ ID NO. 66

```
CAGGTACAGCTGCAGCAGTCAGGACCTGAGCTGGCGAGTCCCGGGGCATCAGTGACACTG
Q  V  Q  L  Q  Q  S  G  P  E  L  A  S  P  G  A  S  V  T  L

TCCTGCAAGGCTTCTGGCGACACACTTACTGACCATATTATGAATTGGGTAAAACAGAGG
S  C  K  A  S  G  D  T  L  T  D  H  I  M  N  W  V  K  Q  R

CCTGGACAGGGCCTTGAGTGGATTGGAAGGATTTATCCAGTAAGTGGTGAAAGTAACTAC
P  G  Q  G  L  E  W  I  G  R  I  Y  P  V  S  G  E  S  N  Y

AATCAAAAGTTCATGGGCAAGGCCACATTCTCTGTAGACCGGTCCTCCAGCACGGTGTAT
N  Q  K  F  M  G  K  A  T  F  S  V  D  R  S  S  S  T  V  Y

ACGGTGTTGAACAGCCTGACATCTGAAGACCCTGCTGTCTATTACTGTGGAAGGGGGGAG
T  V  L  N  S  L  T  S  E  D  P  A  V  Y  Y  C  G  R  G  E

ATCTTTGACTATTGGGGGCAAGGGACCACGGTCACCGTYTCGAGT
I  F  D  Y  W  G  Q  G  T  T  V  T  V  S  S
```

G110 V_L

SEQ ID NO. 67 encoding SEQ ID NO. 68

```
GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTATTGGAGACAGAGTCACC
D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  I  G  D  R  V  T

ATCACCTGCCGGGCCAGTGAGGGTATTTATCACTGGTTGGCCTGGTATCAGCAGAAGCCA
I  T  C  R  A  S  E  G  I  Y  H  W  L  A  W  Y  Q  Q  K  P

GGGAAAGCCCCTAAACTCCTGATCTATAAGGCCTCTAGTTTAGCCAGTGGGGCCCCATCA
G  K  A  P  K  L  L  I  Y  K  A  S  S  L  A  S  G  A  P  S

AGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCT
R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  Q  P

GATGATTTTGCAACTTATTACTGCCAACCATATAGTAATGATGCGCTCACTTTCGGCGGA
D  D  F  A  T  Y  Y  C  Q  P  Y  S  N  D  A  L  T  F  G  G

GGGACACGACTGGAGATTAAACGT
G  T  R  L  E  I  K  R
```

G112 V_H

SEQ ID NO. 69 encoding SEQ ID NO. 70

```
CAGGTCAACTTAAGGGAGTCTGGGGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATC
Q  V  N  L  R  E  S  G  A  E  V  K  K  P  G  E  S  L  K  I

TCCTGTAAGGGTTCTGGATACAGCTTTACCAGCTACTGGATCGGCTGGGTGCGCCAGATG
S  C  K  G  S  G  Y  S  F  T  S  Y  W  I  G  W  V  R  Q  M

CCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGATACCAGATAC
P  G  K  G  L  E  W  M  G  I  I  Y  P  G  D  S  D  T  R  Y

AGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTAC
S  P  S  F  Q  G  Q  V  T  I  S  A  D  K  S  I  S  T  A  Y

CTGTAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGACGGTGG
L  Q  W  S  S  L  K  A  S  D  T  A  M  Y  Y  C  A  R  R  W

AAAGGGCACTTTGACTACTGGGGCCAAGGCACCTTGGTCACCGTCTCGAGT
K  G  H  F  D  Y  W  G  Q  G  T  L  V  T  V  S  S
```

G112 V_L

SEQ ID NO. 71 encoding SEQ ID NO. 72

```
GACATCGTGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACAGAGTCACC
D  I  V  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R  V  T

ATCACTTGCCGGGCCAGTCAGGGTATTAGTAGCTGGTTGGCCTGGTATCAGCAGAAACCA
I  T  C  R  A  S  Q  G  I  S  S  W  L  A  W  Y  Q  Q  K  P

GGGAGAGCCCCTAAGGTCTTGATCTATAAGGCATCTACTTTAGAAAGTGGGGTCCCATCA
G  R  A  P  K  V  L  I  Y  K  A  S  T  L  E  S  G  V  P  S

AGGTTCAGCGGCAGTGGATCTGGGACAGATTTCGCTCTYACCATCAGCAGTCTGCAACCT
R  F  S  G  S  G  S  G  T  D  F  A  L  T  I  S  S  L  Q  P

GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCCCGTGGACGTTCGGCCAA
E  D  F  A  T  Y  Y  C  Q  Q  S  Y  S  T  P  W  T  F  G  Q

GGGACCAAGCTGGAGGTCAAACGT
G  T  K  L  E  V  K  R
```

D5 VH

SEQ ID NO. 73 encoding SEQ ID NO. 74

```
CAGGTGCAGCTGGTGGAGGTCGGAGGCTTGGTCAAGCCTGGAGGGTCCTTAAGAAGTTCT
Q  V  Q  L  V  E  V  G  G  L  V  K  P  G  G  S  L  R  S  S

TCCTGTGCAGCCTCTGGATTCACCTTCAGTGACTACTACATTAGTTGGATCCGTCAGGCT
S  C  A  A  S  G  F  T  F  S  D  Y  Y  I  S  W  I  R  Q  A

CCAGGGAAGGGCTTGAAGTGGGTTGCAAACATTAGTAACAGTGGCAATACCATACACTAC
P  G  K  G  L  K  W  V  A  N  I  S  N  S  G  N  T  I  H  Y

GCAGACTCTGTCAAGGGCCGATTCACCATCTCCAGGGACAACGCCAAGAGGTCAATGTAT
A  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  R  S  M  Y

CTGCAAATGAACAGCCTGAGAGCCGACGACACGGCCGTCTATTACTGTGCGAGGGTGCGA
L  Q  M  N  S  L  R  A  D  D  T  A  V  Y  Y  C  A  R  V  R

TCAATTCGGAATAGTCGCTGGTTCGACCCCTGGGGCCAAGGAACCCTGGTCACCGTCTCC
S  I  R  N  S  R  W  F  D  P  W  G  Q  G  T  L  V  T  V  S

TCG
S
```

D5 VL

SEQ ID NO. 75 encoding SEQ ID NO. 76

```
GAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACATGC
E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T  C

CAAGGAGACAGCCTCAGAAGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACGGGCC
Q  G  D  S  L  R  S  Y  Y  A  S  W  Y  Q  Q  K  P  G  R  A

CCTGTACTTGTCATCTATGGTAAAAACAACCGGCCCTCAGGGATCCCAGACCGATTCTCT
P  V  L  V  I  Y  G  K  N  N  R  P  S  G  I  P  D  R  F  S

GGCTCCAGCTCAGGAAACACAGCTTCCTTGACCATCACTGGGGCTCAGGCGGAAGATGAG
G  S  S  S  G  N  T  A  S  L  T  I  T  G  A  Q  A  E  D  E

GCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAACCATGTGGTATTCGGCGGAGGG
A  D  Y  Y  C  N  S  R  D  S  S  G  N  H  V  V  F  G  G  G

ACCAAGCTGACCGTCCTAGG
T  K  L  T  V  L  G
```

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(22) International Filing Date:
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(74) Agents: **WALTON, Seán, M.** et al.; Mewburn Ellis, York House, Kingsway, London, Greater London WC2B 6HP (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *with international search report*

(88) Date of publication of the international search report:
13 June 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/44300 A3

(54) Title: BRAIN SPECIFIC BINDING MEMBERS

(57) Abstract: The present invention relates to specific binding members directed to the brain, including specific binding members able to pass through the blood brain barrier or which are directed to areas of brain inflammation or blood brain barrier breakdown. Panels and mixtures of antibodies are provided, also individual antibody molecules and VH and VL domains, along with methods of obtaining specific binding members by selection, e.g. using libraries displayed on the surface of filamentous bacteriophage.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04501

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/18 G01N33/50 G01N33/68 G01N33/569 A61K39/395
C12N15/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 00150 A (PARDRIDGE WILLIAM M ;UNIV CALIFORNIA (US)) 7 January 1999 (1999-01-07) abstract page 2, line 12-18 page 5, line 1-18 --- -/--	21-23, 34,36-38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2001	27.07.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montrone, M

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 527 527 A (FRIDEN PHILLIP M) 18 June 1996 (1996-06-18) abstract column 1, line 19-50 column 2, line 5-17 column 4, line 32-47 column 5, line 5 column 5, line 31,32 column 6, line 19-34 column 7, line 17-56 column 12, line 62 -column 13, line 14	21-28, 33,34, 36-38
X	LI JIAN YI ET AL: "Genetically engineered brain drug delivery vectors: Cloning, expression and in vivo application of an anti-transferrin receptor single chain antibody-streptavidin fusion gene and protein." PROTEIN ENGINEERING, vol. 12, no. 9, 1999, pages 787-796, XP000984720 ISSN: 0269-2139 abstract	21-28, 33,34
X	PARDRIDGE WILLIAM M: "Physiologic-based strategies for protein drug delivery to the brain." JOURNAL OF CONTROLLED RELEASE, vol. 39, no. 2-3, 1996, pages 281-286, XP000589688 ISSN: 0168-3659 abstract page 283, column 1, paragraph 2 -page 284, column 1, paragraph 2	21-28,34
X	TUOMANEN ELAINE I ET AL: "Reversible opening of the blood-brain barrier by anti-bacterial antibodies." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 90, no. 16, 1993, pages 7824-7828, XP000984717 Washington, US ISSN: 0027-8424 abstract page 7824, column 1, paragraph 2 page 7826, column 2, paragraph 2 page 7827, column 1, paragraph 4 -page 7828, column 1, paragraph 1	21-28,34

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
Y	WO 97 17613 A (ELAN CORP PLC ;MAHONY DANIEL JOSEPH O (IE)) 15 May 1997 (1997-05-15) abstract page 1, line 8-14 page 2, line 28 -page 3, line 20 page 4, line 35 -page 5, line 20 page 7, line 6-12 page 8, line 29 -page 9, line 7 page 9, line 17-36 ---	5-14,18, 19, 21-28, 31-38
Y	SHEETS MICHAEL D ET AL: "Efficient construction of a large nonimmune phage antibody library: The production of high-affinity human single-chain antibodies to protein antigens" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 95, no. 11, 26 May 1998 (1998-05-26), pages 6157-6162, XP002134533 Washington, US ISSN: 0027-8424 abstract page 6157, column 1, paragraph 2 -column 2, paragraph 3 page 6161, column 2, paragraphs 4,5 ---	5-14,18, 19, 21-28, 31-38
Y	PASQUALINI RENATA ET AL: "Organ targeting in vivo using phage display peptide libraries." NATURE (LONDON), vol. 380, no. 6572, 1996, pages 364-366, XP002128874 ISSN: 0028-0836 abstract page 366, column 1, paragraph 2 -column 2, paragraph 3 ---	5-14,18, 19, 21-28, 31-38
Y	O'SULLIVAN G ET AL: "MONOCLONAL ANTIBODIES TO HUMAN SERUM AMYLOID P-COMPONENT" NATVIG, J. B., ET AL. (ED.). AMYLOID AND AMYLOIDOSIS, 1990;VITH, 1991, pages 906-910, XP000984837 1990. XXVIII+937P. KLUWER ACADEMIC PUBLISHERS: DORDRECHT, NETHERLANDS; NORWELL, MASSACHUSETTS, USA. ILLUS. MAPS. ISBN 0-7923-1089-6. 1991 abstract page 906, paragraph 1 page 909, paragraph 1 --- -/--	5-14, 20-28, 31-35, 37,38

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KALARIA R N ET AL: "SERUM AMYLOID P IN ALZHEIMER'S DISEASE IMPLICATIONS FOR DYSFUNCTION OF THE BLOOD-BRAIN BARRIER" ANNALS OF THE NEW YORK ACADEMY OF SCIENCES,US,NEW YORK ACADEMY OF SCIENCES, NEW YORK, NY, vol. 640, 1991, pages 145-148, XP000603862 ISSN: 0077-8923 abstract page 146, paragraphs 1-3 page 147, paragraph 1 -----	5-14, 20-28, 31-35, 37,38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/04501

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-4, 15-17,29,30
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

As a result of the prior review under R. 40.2(e) PCT,
all additional fees are to be refunded.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-4, 15-17,29,30

Present claims 1 to 4, 15 to 17, 29 and 30 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the antibodies which showed a transmigration across the blood brain barrier: D5, G65, G73, G77 and G93 (see page 62, second para. of the description).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/04501

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9900150 A	07-01-1999	AU 8269898 A	19-01-1999
US 5527527 A	18-06-1996	US 5182107 A	26-01-1993
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